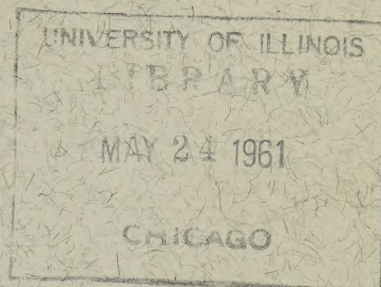


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ERRATUM Vol. 29, No. 4

On p. 449 of the article "A contribution to the rational classification of actinomycetes" by Zh. P. Teshich, in the tabulated information in the summary, the Latin names and the descriptions of Genus V and Genus VI should be interchanged.

HYDROXYLAMINE AND OXIMES AS NITROGEN SOURCES FOR ASPERGILLUS NIGER

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By its chemical composition, hydroxylamine is an intermediate compound between the oxidized and reduced forms of nitrogen. Most molds utilize both forms of nitrogen in synthetic metabolism. It is therefore understandable that physiologists are trying to detect the intermediate products of nitrate reduction, particularly hydroxylamine (Foster, 1950), and to establish its suitability as a nitrogen source.

Raciborski (1907) observed the growth of fungi on nutrient media containing sucrose and hydroxylamine as the nitrogen source. On the basis of his experiments, Raciborski concluded that hydroxylamine is not a universal plasmatic nucleus. However, Loew (1907) did not agree with Raciborski's conclusions; in his opinion, Raciborski did not disprove the view that substances such as hydroxylamine which act on aldehydes and ketones are toxic for all living things, since oximes and hydrazones may have been formed in Raciborski's experiments with sucrose. Lemoigne, Monguillon, and Desveaux (1936) confirmed Raciborski's results and showed that, at a concentration of 0.4 g/liter, hydroxylamine added in 48 hours following inoculation does not inhibit the growth of *Aspergillus niger* on Czapek's medium with ammonium nitrate.

The authors further found that, on media with glycerol instead of sucrose and with hydroxylamine (0.4 g/liter) as the sole nitrogen source, *A. niger* starts to grow later than on ammonium nitrate, but then overtakes the control culture in growth and, at the end of the experiment, after 75 days, the fungus crop is greater on the medium with hydroxylamine than on that with ammonium nitrate, because autolysis of the fungus begins on the latter medium.

Before testing hydroxylamine as a source of nitrogen for *A. niger*, we established the hydroxylamine concentration which is not toxic for this fungus, and also showed the dependence of growth inhibition on the reaction of the nutrient medium.

METHODS

A. niger was grown in the submerged state on a shaker at 30° on nutrient media (30 ml) containing: 1% glucose; 0.3% $(\text{NH}_4)_2\text{SO}_4$; 0.06% KH_2PO_4 (Table 1) (or 0.52% $\text{NH}_4\text{H}_2\text{PO}_4$) and 0.042% K_2SO_4 (Table 2); 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.005% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.003% $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.03% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

The nutrient media were inoculated with conidia to give 500,000 conidia per 1 ml of medium. Day-old mycelium was used as inoculum in experiments Nos. 2 and 3 in Table 2; the amount of mycelium added is indicated in the table. The mycelium was dried to constant weight at 80°.

The hydroxylamine hydrochloride solution was prepared with sterile distilled water immediately prior to the experiment.

Free and bound hydroxylamine was determined by Csáky's method (1948) by oxidation with iodine to nitrous acid, which was then determined with Griess reagent on a photoelectric colorimeter.

The Effect of Different Hydroxylamine Concentrations on the Growth of *A. niger*

When the effect of different hydroxylamine concentrations on the growth of *A. niger* was studied, it was found that, on complete nutrient medium having a pH of 4.2 (the reaction of media usually employed), 0.5 mM/liter of hydroxylamine had no effect on the growth of the fungus; when the concentration was increased fivefold, hydroxylamine inhibited growth by 77.2%, while when the hydroxylamine content was again doubled, growth of the fungus ceased almost entirely (Table 1).

The Effect of Hydroxylamine on the Growth of *A. niger* as a Function of the Reaction of the Nutrient Medium

The intake of substances, especially of weak electrolytes, into the cells of microorganisms depends largely on the reaction of the nutrient medium. Hydroxylamine is a very weak base which dissociates

Table 1. The Effect of Different Hydroxylamine Concentrations on the Growth of *A. niger* (pH 4.2 prior to experiment. Duration of experiment; 24 hours)

Hydroxylamine concentration, mM/liter	Weight of fungus	
	mg	% of control
0	31.1	100.0
0.5	31.2	100.3
2.5	7.1	22.8
5.0	2.3	7.3

Note. We did the work with hydroxylamine in connection with its utilization for trapping activated carboxyl groups, which is reported in another article.

Table 2. The Effect of Hydroxylamine on the Growth of *A. niger* as a Function of the Reaction of the Nutrient Medium

Expt. no.	Expt. duration, hr	Initial pH	Hydroxylamine in nutrient medium, mM/liter	Final pH	Crop of fungus		Hydroxylamine bound in:				not found, μ g
							medium		mycelium		
					mg	% of control	μ g	in % mycelium wt	μ g	in % mycelium wt	
1	24	4.0	None	2.9	37.2	100.0	—	—	—	—	—
		"	2.5	3.1	12.4	33.3	Not determined				
		5.4	None	2.9	46.3	100.0	—	—	—	—	—
		"	2.5	5.2	No growth		Not determined				
2	10	2.8	None	2.7	7.8	100.0	—	—	—	—	—
		"	2.5	2.7	5.9	75.6	222.0	1.6 **	1.2	0.008**	884.8
		5.5	None	5.1	10.3	100.0	—	—	—	—	—
		"	2.5	5.1	0.9	8.6	842.0	9.3**	2.5	0.027**	1311.5
3	20	2.7	None	2.4	61.5	100.0	—	—	—	—	—
		"	2.5	2.4	72.0	118.7	Not determined				
		5.2	None	2.8	68.3	100.0	—	—	—	—	—
		"	2.5	3.1	28.3	41.4	Not determined				

*8.2 mg of mycelium added at inoculation.

**Bound hydroxylamine in medium and in mycelium calculated for mycelium added at inoculation and that produced during experiment, i.e., 14.1 and 9.1 mg.

even less than ammonia. Because of this, we considered it necessary to test the effect of hydroxylamine on the growth of *A. niger* at different acidities of the medium (Table 2).

In the experiments presented in Table 2, hydroxylamine hydrochloride was added in amounts to give 2.5 mM/liter, i.e., 5.208 mg was added to each flask containing 30 ml of nutrient medium. In the first experiment, the nutrient medium had a pH of 4.0 and 5.4 at the start of the experiment, and was inoculated with *A. niger* conidia, while in the second experiment, the nutrient medium had a pH of 2.8 and 5.5 and was inoculated with mycelium. In the latter case, the weight of the fungus is given after the subtraction of mycelium added during inoculation.

It is seen from Table 2 that at pH 4.0, hydroxylamine inhibited growth of the fungus by 66.7%, while at pH 5.4, growth ceased entirely. In the second experiment, at pH 2.8, i.e., at a more acid reaction of the nutrient medium than in the first case, the inhibition of fungal growth by hydroxylamine was weaker and constituted 24.4%, while at pH 5.5, just as in the preceding experiment, inhibition increased nearly fourfold and reached 91.4%.

In the third experiment, a more acid medium (pH 2.7 and 5.2) was used than in the preceding experiments. When the nutrient medium with pH 2.7 was

inoculated with mycelium (8.2 mg), the same concentration of hydroxylamine as that in experiments 1 and 2 did not inhibit growth of the fungus. On medium with pH 5.2, inhibition reached 58.6%.

In the second experiment, aside from the fungus crop and changes in the reaction of the medium, hydroxylamine was also determined at the end of the experiment. Analysis of the nutrient medium and mycelium carried out at the end of the experiment showed that in medium with pH 2.8, 4.100 mg of 5.208 mg of hydroxylamine hydrochloride remained free in the medium, while in the medium with pH 5.5, the amount remaining free was 3.052 mg.

During growth of the fungus on the medium with pH 2.8, 0.222 mg of hydroxylamine was converted to the bound state, while on medium with pH 5.5, the amount bound was 0.842 mg; this constituted 4.2% of the amount added at the start of the experiment in the first case, and 16.1% in the second. Less bound hydroxylamine was also found in the mycelium of the fungus grown at pH 2.8 than in that grown at pH 5.5.

Analyses showed that hydroxylamine is not converted to the bound state in the original nutrient medium having various reactions, nor in culture fluid after the fungus has grown in it. Consequently, hydroxylamine is bound by some substances in the cells of the fungus and is secreted back into the medium in

Table 3. Hydroxylamine as a Nitrogen Source for *A. niger*
(pH prior to experiment No. 1, 4.0; prior to experiment No. 2, 5.0)

Expt. no.	Duration of experiment, hrs	Nitrogen source, mM/liter	pH after experiment	Weight of mycelium	
				mg	in % of control
1	24	0.5 (NH ₄) ₂ SO ₄	1.9	18.3	100
		Without nitrogen	3.2	0.9	4.9
		0.5 NH ₂ OH	3.4	1.0	5.4
2	19	2.5 NaNO ₃	4.9	34.7	100
		Without nitrogen	2.7	2.2	6.3
		2.5 NH ₂ OH	2.8	1.6	6.6

Table 4. The Effect of pH on the Nonenzymatic Conversion of Hydroxylamine
At the start of the experiment, there was 173.7 µg/ml of NH₂OH·HCl at 5°

pH	Decrease in hydroxylamine in:						Accumulation of nitrites in 72 hours		
	100 min		24 hr		48 hr		72 hr		
	γ	% of that added	γ	% of that added	γ	% of that added	γ	% of that added	
								γ	% of hydroxylamine which had disappeared
Water									
2.6	0.0	0.0	6.7	3.8	17.7	10.1	Not determined	None	—
5.0	0.0	0.0	18.4	10.6	—	—		Traces	—
7.0	55.1	31.7	130.4	75.0	157.4	90.6		21.0	12.9
Nutrient medium									
2.5	1.7	0.9	10.1	5.8	—	—	Not determined	None	—
5.0	0.0	0.0	6.1	3.5	17.7	10.1		Traces	—
6.9	33.7	19.4	91.7	52.7	124.4	71.6		25.0	18.0

the bound state. It is evidently bound by some substances which are essential for the normal metabolism of the fungus which are not ordinarily given off into the medium in the absence of hydroxylamine.

After the experiment, having determined free and bound hydroxylamine in the nutrient medium and in the mycelium, we found a 16.0% loss of hydroxylamine in the medium with pH 2.8 and a 25.2% loss in the medium with pH 5.5. As shown by the experiments given below, this loss is possibly explained by the non-enzymatic oxidation of hydroxylamine to nitrous acid.

Hydroxylamine as a Nitrogen Source

As has been pointed out, at pH 4.2, 0.5 mM/liter of hydroxylamine has no harmful effect on the growth of *A. niger*; therefore, hydroxylamine was used in this concentration in experiments where it was the sole nitrogen source. In another experiment, five times more hydroxylamine was added to Czapek's nutrient medium. Lemoigne et al. carried out experiments on Czapek's medium. In Loew's opinion, this medium, which contains 2% invert sugar, caused hydroxylamine to be nontoxic in Raciborski's experiments. The amount of hydroxylamine which we added was 5 and 24 times less, respectively, than that used in the experiments of Lemoigne et al. In the given experiments, nutrient

media containing no nitrogen, as well as those containing ammonium sulfate and sodium nitrate, served as controls.

The results of the experiments summarized in Table 3 show that the growth of the fungus was very slight in the flasks which did not contain nitrogenous substances. Growth of the fungus in cultures containing 0.5 mM/liter of hydroxylamine was the same as the growth on nitrogen-free medium, while with 2.5 mM/liter of hydroxylamine, growth was slightly less. At the same time, on nutrient media with sodium nitrate and ammonium sulfate added to the medium in the same amounts as hydroxylamine, the crop of fungus was 15–20 times larger than without nitrogen or with hydroxylamine. This experiment shows that *A. niger* does not utilize free hydroxylamine as a nitrogen source on media containing glucose and invert sugar. Thus, our results do not agree with those of Raciborski or those of Lemoigne et al.

As shown by our experiments which are given below, the reason for the divergence in results may possibly consist of the fact that in the experiments of Lemoigne et al., hydroxylamine was oxidized to nitrous acid and, consequently, the fungus may have grown at the expense of the nitrous acid produced.

Nonenzymatic Oxidation of Hydroxylamine to Nitrous Acid

As has been pointed out earlier, hydroxylamine, which is an intermediate substance between the oxidized and reduced forms of nitrogen, is easily converted to both forms of nitrogen.

In order to determine the rate of conversion of hydroxylamine by the nonenzymatic pathway, we prepared solutions of hydroxylamine hydrochloride calculated to give 2.5 mM/liter in the nutrient medium used for growing fungi and in water. These solutions were adjusted to the reactions indicated in Table 4 with sulfuric acid or alkali. The solutions were kept in a refrigerator at 5°. Then, after certain intervals of time, the hydroxylamine in them was determined by Csáky's method (1948).

It is seen from Table 4 that the hydroxylamine content in water and in nutrient medium decreased insignificantly at pH 2.6–2.5 and pH 5.0. At a neutral reaction (pH 6.9 and 7.0), on the contrary, the disappearance of hydroxylamine occurred very rapidly. At pH 2.5–5.0, for example, almost no conversion of hydroxylamine was observed in 100 minutes, or else it was very insignificant (a total of 0.9% of the initial amount), while in the same amount of time at pH 6.9, the hydroxylamine content decreased by 19.4%, and by 31.7% at pH 7.0. During 48 hours at pH 2.5–2.6 and 5.0, the hydroxylamine content decreased by only 10%, whereas at pH 6.9, it decreased by 71.6%, and by 90.6% at 7.0. It is striking that, although the difference in reaction of the medium was very small (only 0.1 pH), the difference in conversion rate constituted 19% in 48 hours.

At the end of the experiment, i.e., after 72 hours, we determined nitrous acid in the hydroxylamine solutions; however, the amount of nitrous acid proved to be considerably less than the amount of hydroxylamine which had disappeared. The inconsistency found between the amount of hydroxylamine which had disappeared and nitrous acid formed is probably explained by a reaction between hydroxylamine and nitrous acid leading to the conversion of both substances to molecular nitrogen. Frear and Burrell (1955) also observed a loss of hydroxylamine in plant tissue extracts.

On the basis of this experiment, it can obviously be assumed that the growth of *A. niger* in the experiments of Lemoigne et al. occurred due to the nitrites produced during the oxidation of hydroxylamine. The delay in growth of the fungus observed by Lemoigne et al. at the start of the experiments is evidently explained by the fact that the fungus cannot utilize hydroxylamine as a nitrogen source until it is oxidized to nitrous acid, at which point fungal growth occurs.

No such phenomenon was observed in our experiments, because the experiments were conducted at an acid reaction for 1–2 days.

The fact that *A. niger* is capable of utilizing nitrites as a nitrogen source at a slightly acid reaction has been known for a long time (Kostychev and Tsvetkova, 1956; Foster, 1950, p. 478).

In addition to free hydroxylamine, we also tested bound hydroxylamine in the form of the oximes of pyruvic and α -ketoglutaric acids as nitrogen sources.

The Oximes of Pyruvic and α -Ketoglutaric Acids as Nitrogen Sources for *A. niger*

In order to resolve the question of whether or not the oximes of pyruvic and α -ketoglutaric acids are toxic for *A. niger*, we conducted an experiment in which hydroxylamine (final concentration 2.5 mM/liter), and pyruvic or α -ketoglutaric acid (to give 10 mM/liter) were simultaneously added to complete nutrient medium.

The results of the experiment summarized in Table 5 show that, as in the previously given experiments, hydroxylamine in a concentration of 2.5 mM/liter of medium inhibited the growth of *A. niger* by 90.0–97.8%. Inhibition of fungal growth was not observed on complete nutrient media containing the oximes of pyruvic or α -ketoglutaric acids, while the free keto acids increased the yield of fungus. Consequently, it can be concluded from this experiment that the oximes of pyruvic and α -ketoglutaric acids are not toxic for *A. niger*.

Table 5. The Effect of Hydroxylamine and the Oxime of Pyruvic and α -Ketoglutaric Acids on the Growth of *A. niger* (Duration of experiment 44 hours)

Expt. No.	pH prior to experiment	Added to nutrient medium	pH after experiment	Weight of fungus	
				mg	% of control
1	3.9	Nothing	2.8	28.9	100.0
	3.9	2.5 mM/1 NH ₂ OH	3.4	2.9	10.0
	4.0	10 mM/ liter of pyruvic acid	3.4	38.4	132.8
	4.0	10 mM/ liter of pyruvic acid + 2.5 mM/ liter NH ₂ OH	3.0	28.1	97.2
2	3.9	Nothing	2.9	22.2	100.0
	3.9	2.5 mM/1 NH ₂ OH	3.5	0.5	2.2
	3.9	10 mM/ liter of α -ketoglutaric acid	3.9	33.2	149.5
	3.9	10 mM/ liter of α -ketoglutaric acid + 2.5 mM/ liter NH ₂ OH	3.9	25.8	116.2

Table 6. Hydroxylamine and the Oximes of Pyruvic and α -Ketoglutaric Acids as Sources of Nitrogen for *A. niger* (Duration of experiment No. 1, 23 hours; No. 2, 24 hours)

Expt. No.	pH prior to experiment	Nitrogen sources	pH after experiment	Weight of fungus	
				mg	in % of control
1	3.8	100 mM/1(NH ₄) ₂ SO ₄	2.2	119.8	100
	2.5	2.5 mM/1 NH ₂ OH	2.4	1.3	1.1
	2.6	2.5 mM/liter of pyruvic acid oxime	2.4	1.6	1.3
	3.8	The same	3.0	1.6	1.3
	5.9		3.6	1.5	1.2
2	4.0	1.25 mM/1 (NH ₄) ₂ SO ₄	2.7	18.9	100
	4.2	Without nitrogen	4.1	1.4	7.4
	3.9	2.5 mM/1 NH ₂ OH		0.7	3.7
	3.3	2.5 mM/liter of α -ketoglutaric acid oxime		2.4	12.7

According to the hypothesis of Bakh and Meier (1937) and Schultz, the hydroxylamine which is produced in plants during the reduction of nitrates reacts with keto acids; the oximes produced in this manner are then reduced to the corresponding amino acids. Kretovich, Bundel', Frasheri, and Borovikova (1958) point out that in a leaf slurry of pumpkin and wheat, glutamic acid and serine are formed, possibly by means of the reduction of the corresponding oximes, or else hydroxylamine is first reduced to ammonia which then reacts with keto acids.

After we were able to establish that the oximes of α -keto acids are not toxic for *A. niger*, we tried to determine whether these oximes are sources of nitrogen for the fungus under investigation.

For this purpose, an experiment was conducted in which the oximes of α -ketoglutaric and pyruvic acids were used as the nitrogen source; the oxime of α -ketoglutaric acid was tested at pH 4.0, while the oxime of pyruvic acid was tested at pH 2.6, 3.8, and 5.9.

It is seen from Table 6 that the growth of *A. niger* on medium containing no nitrogen except α -ketoglutaric acid oxime was very slight, almost equaling the growth of the fungus on nitrogen-free medium. The same poor growth of the fungus was also observed on pyruvic acid oxime at all of the reactions tested.

On the basis of the results obtained, it can be concluded that *A. niger* does not utilize the oximes of α -ketoglutaric and pyruvic acids as nitrogen sources and that they are probably not reduced to the corresponding amino acids.

As has been pointed out above, at a neutral reaction, hydroxylamine is oxidized to nitrites quite rapidly by the oxygen in the air. As far as the oximes of α -ketoglutaric and pyruvic acids are concerned, these are not oxidized by oxygen from the air at acid and neutral reactions.

SUMMARY

1. The toxic effect of hydroxylamine on the growth of *Aspergillus niger* depends on its concentration in the nutrient medium and on the reaction of the medium.

2. At pH 4.2, 0.5 mM/liter of hydroxylamine does not inhibit the growth of *A. niger*. When the concentration is increased fivefold, inhibition reaches 77.2%, while at a hydroxylamine concentration of 5 mM/liter, growth of the fungus ceases almost completely.

3. In nutrient medium which has an initial pH of 2.7 and a final pH of 2.4, hydroxylamine in a concentration of 2.5 mM/liter does not inhibit the growth of *A. niger*. At the same time, at a pH of the medium of 5.5, the same concentration of hydroxylamine inhibits fungal growth completely.

4. Hydroxylamine is bound in *A. niger* cells by some substances which are necessary for normal metabolism of the fungus and are not released into the nutrient medium in the absence of hydroxylamine.

5. Hydroxylamine is not utilized by *A. niger* as a nitrogen source.

6. Hydroxylamine is oxidized to nitrous acid by a nonenzymatic pathway in aqueous solutions. When the pH of the solutions was 2.5-5.0, hydroxylamine decreased by 10% in 48 hours, while at pH 7.0, it decreased by 90.6% of the initial amount.

7. In amounts of 2.5 mM/liter, the oximes of pyruvic and α -ketoglutaric acids do not inhibit the growth of *A. niger*.

8. At pH 2.5-5.9, the oximes of pyruvic and α -ketoglutaric acids are not utilized as nitrogen sources by *A. niger*.

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THE OXIDATION OF GLUCOSE BY A CULTURE OF ACTINOMYCES

VIOLACEUS, STRAIN 719

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The study of the breakdown of carbohydrates led to the determination of a great number of intermediate products, among which were found hexose phosphate, triose phosphate, phosphopyruvic acid, etc. Pyruvic acid is the initial substance for a large number of very important conversions in the metabolism of the living cell, and the conversion of pyruvic acid proceeds differently in different microorganisms.

Shen et al. (1956), Sih (1956, 1957), and others have done work in the field of the enzymes and intermediate products of carbohydrate metabolism in actinomycetes. Sih found all of the enzymes participating in the hexose monophosphate cycle of carbohydrate conversion according to the Embden-Meyerhof-Parnas scheme (with the exception of phosphohexokinase) in the cell-free centrifugate of 48-hour mycelium of strain No. RRL-1951 B-25.

Shen's work (1956) was devoted to the first stage of carbohydrate breakdown in *Streptomyces aureofaciens* and to the effect of orthophosphate on carbohydrate metabolism. Shen showed that *Streptomyces aureofaciens* can carry out carbohydrate conversions by two pathways, depending on the content of orthophosphate in the medium. Mycelium grown on medium with a low orthophosphate content metabolizes carbohydrates chiefly through hexose monophosphate, while mycelium grown on phosphate-enriched medium breaks down carbohydrates by the Embden-Meyerhof-Parnas scheme with the production of pyruvic acid; both of these pathways are linked through a common intermediate product: fructose-1,6-diphosphate.

From the data given, it is seen that those enzyme systems have been studied which decompose carbohydrates to pyruvic acid.

The purpose of the present work is to study the intensity of glucose oxidation by a culture of *Actinomyces violaceus* and to determine the organic acids which are produced by this microorganism as the result of the conversion of glucose.

EXPERIMENTAL METHODS

The *A. violaceus* fermentation was carried out on synthetic medium which included the following in its composition (in %): glucose-2; K_2HPO_4 -0.05; $MgSO_4$ -0.05; NaCl-0.05; KNO_3 -0.1; $CaCO_3$ -0.25; traces of iron.

In the majority of cases, a young culture was used to study the respiratory processes of the microorganism which we were investigating. Washed mycelium was placed in phosphate buffer at pH 7.0, and sterile air was passed through it for 12 hours.

The mycelium was washed with water several times and was then filtered, weighed, and a known amount of it was transferred to a buffer solution at pH 7.0.

The effect of glucose on the respiration of *A. violaceus* was studied in Warburg respirometers. The scheme of the experiments was as follows: The test suspension containing 100 mg of mycelium and 3 ml of phosphate buffer (pH 7.0) was placed in the vessel. Glucose solution (0.5 ml, 0.5%) was placed in the side-arm, and after the respirometers were equilibrated to constant temperature, the glucose was tipped into the vessels. In cases where oxygen consumption was being measured, 0.2 ml of 20% KOH was placed in the center well, while in cases where carbon dioxide evolution was being measured, water was used. A mycelial suspension of the test culture, but no sub-

Table 1. The Effect of Glucose on the Respiration of *A. violaceus*
(0.2 g of mycelium in 3 ml of phosphate buffer at pH 7.0; glucose concentration—0.5%,
duration of experiment—3 hours)

Experimental variant	Oxygen consumption in μ liters from start of experiment after (min)						
	30	60	90	120	150	180	3-hour total
Culture	87.6	79.2	72.0	78.4	68.4	66.0	451.2
Culture + glucose	137.6	148.0	158.4	166.0	144.6	169.9	797.4
Carbon dioxide evolution in μ liters							
Culture	33.6	32.0	27.2	27.2	27.2	35.2	182.4
Culture + glucose	43.7	63.0	78.0	86.4	99.0	153.0	522.4

Table 2. Changes in the Gas Exchange Coefficient in *A. violaceus*

Experimental variant	30 min	60 min	90 min	120 min	150 min	180 min	In 3 hr (average)
Culture	0.38	0.40	0.38	0.35	0.40	0.5	0.40
Culture + glucose	0.27	0.42	0.49	0.52	0.65	0.9	0.56

strates, was placed in control vessels. The experiments were carried out at 25°.

The results of the analyses are given in Tables 1 and 2.

The course of oxygen consumption and carbon dioxide evolution is given in Table 1. The intensity of oxygen consumption by the culture decreased with time when substrate was not added. Thus, for example, whereas the culture consumed 87.6 μ liters in the first 30 minutes, after 2.5 hours it consumed 66.0 μ liters in 30 minutes. It can be assumed that the endogenous material in the cell is used up for respiration, and the amount of oxygen consumed therefore decreases.

Carbon dioxide evolution fluctuated very little during these intervals of time, and therefore the respiratory quotient did not change as much. The average respiratory quotient for three hours was 0.4 (Table 2).

In the presence of glucose, an increase in the amount of oxygen consumed (from 137.5 μ liters at the start to 169.9 μ liters of O₂ at the end of the experiment) and a simultaneous increase in carbon dioxide evolution (from 43.7 μ liters of CO₂ at the start to 153 μ liters of CO₂ at the end of the experiment) were observed.

The average respiratory quotient in the presence of glucose was 0.56 (Table 2). This respiratory quotient shows that the process of glucose oxidation did not proceed to complete combustion—to carbon dioxide and water—since in this case the respiratory quotient should have been equal to 1.

This reduced respiratory quotient shows that the microorganism which we tested does not oxidize glucose completely; unknown reduced substances are formed from it from which proteins, lipoids, antibiotics, and other intracellular substances are evidently synthesized. In a recently published work, we showed that *A. violaceus* forms amino acids from glucose and KNO₃ (Bekhtereva, 1958).

It is seen from Table 1 that when glucose is added to the mycelium, it is first oxidized, and then the oxidized products are decarboxylated and carbonyl compounds are produced at the same time. Investigations showed that these carbonyl compounds form hydrazones with 2,4-dinitrophenylhydrazine. We isolated pyruvic acid hydrazones with a melting point of 218° and acetaldehyde hydrazones with a melting point of 168°, and a number of other still-unidentified hydrazones.

Pyruvic acid was additionally determined by the Frideman and Haugen method (1943).

The aerobic character of growth of *A. violaceus* and the gradual changes in the value of the respiratory quotient in the solutions make it reasonable to suppose that this organism converts glucose through organic acids.

In order to confirm this hypothesis, the formation of organic acids in glucose-containing medium was studied. Ion-exchange resins and the paper chromatography method were used for isolating and identifying organic acids from the culture fluid. The culture fluid (in 100-ml amounts) was passed through a column of cation-exchange resin, after which the solution contained free organic and inorganic acids. The organic acids could be separated from the inorganic ones by repeated extraction with diethyl ether. The extracts obtained were concentrated to 2 ml. For chromatography of the organic acids, 15 μ liters of the test solution was placed on No. 2 Leningrad brand paper. A mixture of n-butyl alcohol (saturated with water) and formic acid in a ratio of 95:5 was used as the solvent. Bromcresol green or bromcresol blue served as developer. Examples of chromatograms are given below (Figs. 1, 2, 3).

As seen from the chromatograms, a large amount of organic acids was found in the culture fluid during *A. violaceus* fermentation; moreover, the amounts of acids were greater in the three- and nine-day cultures than in the six-day one. This was also confirmed when total acidity was determined by titration with potassium hydroxide. The fluid was first passed through SBS brand cation-exchange resin to convert organic acid salts to free acids. Potassium hydroxide solu-

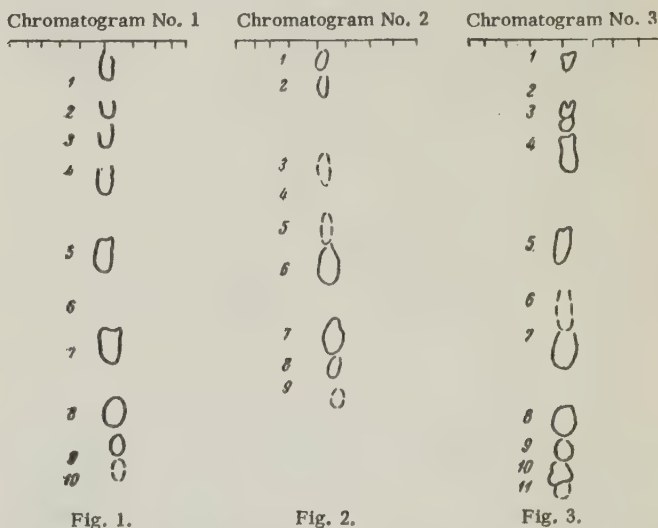


Fig. 1.

Fig. 2.

Fig. 3.

Figs. 1-3. Chromatograms Nos. 1-3. The production of organic acids during the growth of an *A. violaceus* strain 719 culture on medium with glucose.

Chromatogram No. 1. Three-day culture. 1) Oxalic acid; 2) gluconic; 3-4) unknown acids; 5) α -ketoglutaric; 6) malic; 7) glycolic; 8) lactic; 9) pyruvic; 10) succinic.

Chromatogram No. 2. Six-day culture. 1) Gluconic acid; 2) unknown; 3) α -ketoglutaric; 4) malic; 5) glycolic; 6) lactic; 7) pyruvic; 8) succinic.

Chromatogram No. 3. Nine-day culture. 1) Oxalic acid; 2) gluconic; 3-4) unknown; 5) α -ketoglutaric; 6) malic; 7) glycolic; 8) lactic; 9) pyruvic; 10) succinic; 11) fumaric.

tion (13.1 ml, 0.1 N) was used to neutralize 50 ml of three-day culture fluid. Ten ml was required for the six-day culture fluid, and 11.5 ml of 0.1 N KOH for the nine-day culture fluid.

As can be seen, organic acids underwent quantitative changes during the course of growth of *A. violaceus*. The amount of lactic acid changed noticeably as the culture developed. The largest amounts of lactic acid were produced in the three-day culture (86.4 mg/liter), it decreased noticeably in the six-day culture (40 mg/liter) and, finally, an increase in lactic acid was observed in the nine-day culture (68 mg/liter).

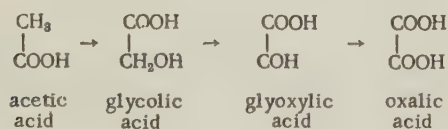
The given microorganism produces a number of organic acids from glucose: oxalic, gluconic*, α -ketoglutaric, malic, glycolic, lactic, pyruvic, succinic, fumaric, and three other acids which we have not identified.

It is interesting to recall that *A. violaceus* forms nearly as many acids from pyruvic acid (Bekhtereva, 1960). We were unable to detect citric acid at any developmental phase of the culture. This microorganism does not produce citric acid from pyruvic and oxalacetic acids.

From the results which we obtained, it can be assumed that in *A. violaceus* the breakdown of glucose takes place through pyruvic acid as well as through other carbonyl compounds which have not yet been identified. Further, part of the pyruvic acid is oxidized through the dicarboxylic acid cycle, while part is reduced to lactic acid. This can explain the production of malic, succinic, and lactic acids.

Pyruvic acid can be decarboxylated to acetaldehyde, or acetic acid is produced by the oxidative decarboxylation pathway. We identified acetaldehyde in the form of the 2,4-dinitrophenylhydrazone in the three-day culture of *A. violaceus*, while of the volatile acids, we detected acetic acid.

Acetic acid can also be oxidized to oxalic acid through glycolic and glyoxylic acids, which occurs in certain molds.



All of the acids enumerated in this scheme were detected in the *A. violaceus* culture fluid with the exception of glyoxylic acid. The microorganism investigated by us possibly oxidizes acetic acid according to the scheme indicated above and through the dicarboxylic acid cycle.

SUMMARY

1. A study of the respiration of *A. violaceus* showed that washed and aerated mycelium consumes oxygen without the addition of substrate and that the intensity of respiration decreases with time. The respiratory quotient averaged about 0.4. In the presence of glucose, an increase in oxygen consumption and a simultaneous increase in carbon dioxide evolution were observed. In the presence of glucose, the respiratory quotient was about 0.56.

2. The respiratory quotient in the presence of glucose was nearly 40% below the theoretical, which is evidence of the fact that glucose is not completely oxidized to carbon dioxide and water, but that unknown reduced substances are produced from which amino acids, proteins, lipoids, antibiotics, and other intracellular substances are synthesized.

3. *A. violaceus* first oxidizes glucose and then decarboxylates the oxidized product and forms carbonyl compounds. These carbonyl compounds form hydrazones with 2,4-dinitrophenylhydrazine. We isolated the hydrazones of pyruvic acid with a melting point of 218° and the hydrazones of acetaldehyde with a melting point of 168°, as well as a number of other still unidentified hydrazones.

4. The aerobic character of *A. violaceus* and the gradual changes in the respiratory quotient in solutions suggest that this microorganism transforms glucose via organic acids.

5. *A. violaceus* produces a number of organic acids from glucose, such as oxalic, gluconic, malic, succinic, fumaric, lactic, pyruvic, α -ketoglutaric and three acids not yet identified. This microorganism produces as many acids from pyruvic acid alone. Citric acid has not been detected at any developmental stage.

6. It may be suggested from the evidence obtained in the present study that glucose breakdown in *A. violaceus* proceeds via pyruvic acid as well as some other carbonyl compounds not yet identified. Subsequently, pyruvic acid is oxidized via the dicarboxylic acid cycle, part of it being reduced to lactic acid.

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*Gluconic acid has not yet been definitely identified, since in some experiments its R_f value did not agree with that of pure gluconic acid.

[†]See English translation.

THE EFFECT OF PROLONGED ACTION OF SOME CHEMICAL AND PHYSICAL FACTORS ON THE RADIOSENSITIVITY OF MICROORGANISMS

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The study of the possibility of effecting a stable increase in the radioresistance of an organism is of great practical and theoretical significance.

High resistance to the effect of ionizing radiations has been established in infusoria (Lozina-Lozinskii and Aleksandrov, 1959) and in microorganisms isolated from mineral springs with increased radioactivity. Increased radioresistance of microorganisms acted upon repeatedly by various types of ionizing radiations has been demonstrated by Maisin et al. (1956) and by Pershina (1957).

By irradiating a sensitive strain of *E. coli* with ultraviolet rays, Witkin (1947) succeeded in obtaining a mutant which was highly resistant to radiation and which also proved to be resistant to the action of penicillin.

Microorganisms are a particularly convenient experimental material for the radiobiologist. We therefore felt it would be interesting to use them for studying the possibility of increasing the radioresistance of experimental cultures of microorganisms through prolonged action on them of certain types of ionizing radiations, ultraviolet rays, hydrogen peroxide, and embichin. We expected to obtain microbial variants which are highly resistant to these factors and to compare their radioresistance to that of the original cultures. This would enable us to determine the role of hydrogen peroxide in the phenomenon of the inactivation of microorganisms when they are irradiated in an aqueous medium, as well as to obtain certain materials pertaining to the question of the mechanism and specificity of action of ionizing radiations on live cells.

MATERIALS AND METHODS

Four museum strains of *Bacillus faecalis alcaligenes* were used as experimental materials. The cultures were typical in their properties and differed from one another only in morphology, viability, and certain details of metabolism. The strains were designated K, 415, M, and 4.

During adaptation to hydrogen peroxide and embichin, the cultures were grown on meat-peptone broth containing constantly increasing amounts of these substances. Control inoculations were made after every 48 hours simultaneously with transfers to fresh nutrient medium. We obtained the necessary concentrations of

hydrogen peroxide by diluting 28-30% perhydrol solution, the activity of which was determined by iodometric titration. Embichin solutions were prepared from the dry preparation in ampoules.

From 70 to 100 passages were made during adaptation to these factors. When adaptation was completed, the sensitivity of the experimental and original cultures to the action of hydrogen peroxide and embichin was determined simultaneously by the growth inhibition effect when broth containing various amounts of preparation was inoculated with a known amount of culture.

At the same time, the cultures were subjected to prolonged or repeated treatment with ionizing radiations and ultraviolet rays for the purpose of altering them and obtaining radioresistant variants from them.

We studied the prolonged effect of small doses of radiation on the growing cells of microorganisms by using the β -radiation of Sr^{90} . The radiation source was placed in an incubator where the experimental strains were grown in test tubes of meat-peptone broth for 48 hours at 35.5° at a given distance from the emitter. The hard β -radiation of radioactive strontium (Sr^{90}) was filtered through aluminum foil and the walls of matched standard test tubes. The magnitude of the ionizing radiation dose in the test tube was 1.66 rad/minute in this case. After two days, when new tubes of broth were inoculated, control inoculations were made on solid nutrient media. Each strain underwent 100 transfers with cultivation under conditions of irradiation. In this time, the cultures each received about 478,000 rad. The average number of viable cells contained in 1 ml of nutrient medium at the time of inoculation and after 48 hours of growth of the continuously irradiated culture was determined periodically by the limiting dilutions method and by inoculation into agar. Even by obviously low estimates, not counting the cells which were damaged by radiation and died during the incubation period, we were able to follow the effect of small doses of radiation on more than a thousand generations of microorganism cells.

For adaptation to x-rays, we used the method of irradiating resting cells with sublethal doses of radiation with subsequent plating and accumulation of the progeny of the surviving, more radioresistant cells.

An RUM-7 apparatus served as the source of x-rays. Microbes were irradiated without a filter and without a cone, at a voltage of 50 kv and current strength of

10 ma. The layer of half-reduction under these conditions was 0.05 mm of aluminum.

A cell suspension of the experimental cultures in physiological saline solution was placed in special small, sterile, metal cups covered with cellophane and was irradiated with a specific dose of x-rays. A measured amount of each irradiated suspension was plated on Petri dishes of meat-peptone agar, and these were placed in an incubator for 48 hours. After this period of time, a new cell suspension in saline was prepared from the colonies which grew up and was in turn irradiated with increased doses of x-rays. The increase in the dosage depended on the number of cells which had remained alive in the preceding irradiation of the suspension. Our cultures underwent 28-30 x-ray irradiations, receiving four to twelve million $\underline{r}$ in this period of time.

In studying the effect of ultraviolet radiation on microorganisms, in distinction from the methodology employed in the work with x-rays, Petri dishes of meat-peptone agar were inoculated with a measured amount of the experimental culture (fifty million cells) and were then irradiated with ultraviolet. In all other respects, the methodology did not differ from that described above. The experimental cultures were irradiated 85-100 times.

The radioresistance of the variants obtained and of the original strains was determined by their survival when irradiated with x-rays in an RUM-7 apparatus at the same operating conditions of the apparatus as had been used for the adaptation experiments. For this purpose, 24-hour-old cultures of microorganisms grown on solid nutrient medium were washed off with physiological saline solution, agitated on a shaker for 10-15 minutes, washed once, and diluted with saline to a suspension thickness corresponding to an optical standard of 500 million bacteria. The cell suspension was dispensed in 4-ml amounts in special, sterile, previously paraffined small metal cups (the diameter of the small cups was equal to the diameter of the opening of the tube—4.0 cm, height—1.4 cm). The small cups were covered with sterile cellophane and were placed on a strictly horizontal surface to avoid slanting and alteration of the thickness of the layer (0.32 cm) of the fluid being irradiated and the disturbance of the even absorption of radiation energy con-

nected with this. Irradiation was carried out at 18-20° in an atmosphere of air. The distance from the surface of the liquid to the anode was 4.83 cm. The magnitude of the x-ray dosage in the cups determined by means of a KRM-1 dosimeter was 34,000 r/minute under these conditions. Following irradiation, three or four different dilutions were prepared from the experimental and control suspensions, measured amounts of which were plated on Petri dishes of agar.

After 48 hours, the average number of viable cells per 1 ml of the irradiated and control suspensions was determined by counting the colonies which had grown on the plates. The semilogarithmic curves given in the tables of the survival of experimental cultures acted upon by various doses of x-rays were determined according to the formula:

$$a = \log \frac{n}{N},$$

where n is the number of viable cells contained in a given volume of irradiated suspension, and N is the number of cells in unirradiated suspension. Cell survival was determined over a dosage range of from 3,000 to 408,000 r. In those cases where the survival curves of the experimental variants did not differ from those of the original cultures, they were determined once. In all other cases, as well as with original cultures, experiments of survival determination were repeated.

The resistance of our cultures to ultraviolet rays was also determined by their survival when a suspension of microorganisms was acted upon by various doses of ultraviolet rays. In distinction from the above, when this series of experiments was set up, suspensions of the original strain and of the adapted variant were simultaneously irradiated from a single source for purposes of greater accuracy. In order to avoid photoreactivation, all irradiation suspensions of microorganisms were placed in the dark during determinations of survival curves.

Identical experimental conditions throughout the entire work prevented effects from other unforeseen factors, to some extent.

RESULTS

The course of adaptation of the experimental cultures to the action of some of the factors used is represented

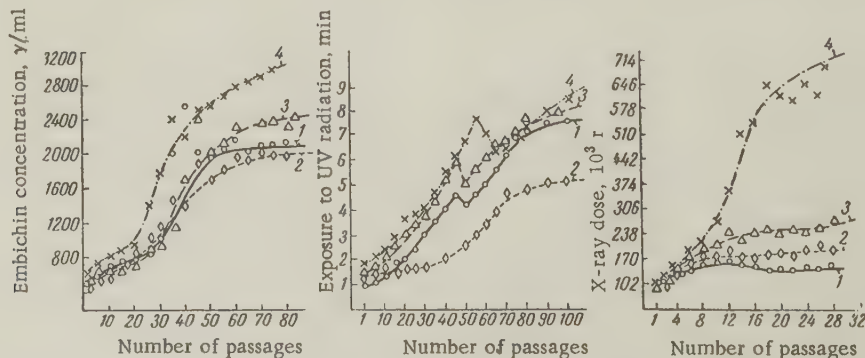


Fig. 1. The course of adaptation of *B. faecalis alcaligenes* to embichin and to ultraviolet and x-rays. 1) Strain K; 2) strain 415; 3) strain M; 4) strain 4.

in Fig. 1. Adaptation was started with concentrations of active substances and radiation doses not exceeding one-half the bactericidal dose. Later, we used the active agents in less than bactericidal doses; therefore, when they were exceeded by the slightest amount due to retardation or absence of growth of the adapted culture, we had to go back to the preceding transfers. Even in the first passages during adaptation to these agents, using dosages close to bactericidal, variants appeared which differed from the original cultures in character of growth on solid nutrient media and in microscopic and serological characteristics. Carbohydrate activity proved to be a stable characteristic which changed little in the experimental cultures treated with ionizing radiations and the other factors enumerated.

B. faecalis alcaligenes does not ferment carbohydrates in Hiss media. In our strains, increased resistance to the physical and chemical agents used was not accompanied by changes in these properties either. Colonies appearing during the course of adaptation which were nontypical in their resistance to the inducing factor did not differ substantially from the original population.

Adaptation to highly active chemical and physical factors occurs extremely slowly in distinction from training to antibiotics which was studied earlier with these same cultures (Kashkin, 1959). However, this process still occurred more easily in strains 4 and M.

Strain K did not adapt at all to some factors (x-rays, hydrogen peroxide), while in the work with other factors, on the contrary, it was not greatly surpassed by strains 4 and M (ultraviolet radiation). Strain 415 adapted to the action of antibiotics better than the others, but adapted to the effect of active chemical and physical factors slowly and with great difficulty. As the result of the experiments carried out, the variants obtained began to tolerate higher concentrations of hydrogen peroxide and embichin. Thus, for strain M, the bactericidal dose of hydrogen peroxide rose 2.5 times as compared with the original culture. An increase in resistance of 1.5 and 1.7 times was noted for strains 415 and 4, respectively. During adaptation to embichin, the greatest increase in resistance (two-fold) was obtained in variants of strains K and M. In strains 4 and 415, sensitivity to the effect of embichin decreased by 1.9 and 1.8 times, respectively.

As the result of repeated treatments of the experimental cultures with ultraviolet radiation, the resistance to this factor, calculated on the basis of LD₅₀, increased 2, 1.8, and 1.4 times in strains K, M, and 4, respectively.

In our experiments, the activity of the β -radiation of Sr⁹⁰ was not great enough to exert a bactericidal effect on the microbial cell.

It is well known (Nadson, 1935; Timofeev-Resovskii, 1956; Sokurova, 1956, 1957a, b and others) that ionizing radiation can have either an invigorating effect; stimulating the processes of growth and metabolism, or, on the contrary, an inhibiting effect on the microbial cell, depending on the magnitude of the dose and radiation intensity. It was therefore interesting to study the growth characteristics of the original cultures and

of those subjected to prolonged irradiation when treated with Sr⁹⁰ β -radiation and without it. Irradiation with Sr⁹⁰ retarded cell division in strains K, M, 415, and 4, inhibiting the growth of these cultures in meat-peptone broth. This was not found in variants of strains 4 and M, which had been subjected to prolonged cultivation under conditions of increased radiation level: Their growth curves under these conditions approximated those of the original cultures without the effect of ionizing radiation. When the variant of strain 4 which was adapted to the β -radiation of Sr⁹⁰ was grown in meat-peptone broth without irradiation, it showed even faster growth and had a higher concentration of cells than the original culture under the same conditions. Without irradiation, the growth of the variant of strain 415 which was adapted to the β -radiation of Sr⁹⁰ did not differ from either the growth of the original culture or that of the same variant grown under the effect of β -radiation. In strain K, prolonged treatment with radioactivity did not lead to increased radioresistance: When irradiated with Sr⁹⁰, the growth curve of this variant did not differ from that of the original culture acted upon by ionizing β -radiation.

When the effect on microorganisms of repeated x-ray irradiation was studied, the individual peculiarities of the strains were most clearly manifested. Whereas rapid adaptation to this treatment was noted in strain 4, it was not possible to increase the bactericidal dosage of x-rays in strain K. X-rays had a clearly pronounced bactericidal effect on strain 415—inoculated plates remained completely sterile after 24 hours of incubation, and growth appeared only after 48 hours.

The radioresistance of the variants obtained by these methods of treatment and of the original cultures was studied in relation to x-rays (Figs. 2, 3, 4). Despite some differences in the properties of the original cultures, their resistance to x-rays was of approximately the same level, and a dose of 170,000–200,000 r was bactericidal for a bacterial suspension of 500 million cells per 1 ml in density (slight fluctuations in the density of the irradiated suspensions did not affect their survival). At doses of 34,000–40,000 r, the course of the survival curve in these cultures changed somewhat, showing a different dependence on the magnitude of the dose in the exponential equation of the curve. We obtained high resistance to x-rays in strain 4 by treating it with the same radiation (Fig. 4). The survival of this variant exceeded the survival of the original strain by many times when irradiated with various doses of radiation. Similar variants of strains 415 and M also became more resistant to large doses of x-rays. In some cases (strains 4 and M), increased resistance to ultraviolet irradiation was accompanied by greater survival of cells when irradiated with x-rays in doses of from 100 to 300 thousand r (Figs. 3 and 4). In strains 415 and 4, which had been subjected to prolonged growth under the effect of Sr⁹⁰, a deviation in the course of the survival curve was noted, demonstrating their higher resistance to the effect of large doses of x-rays as compared with the original cultures. None of the variants of strain K differed from the original cultures in resistance to

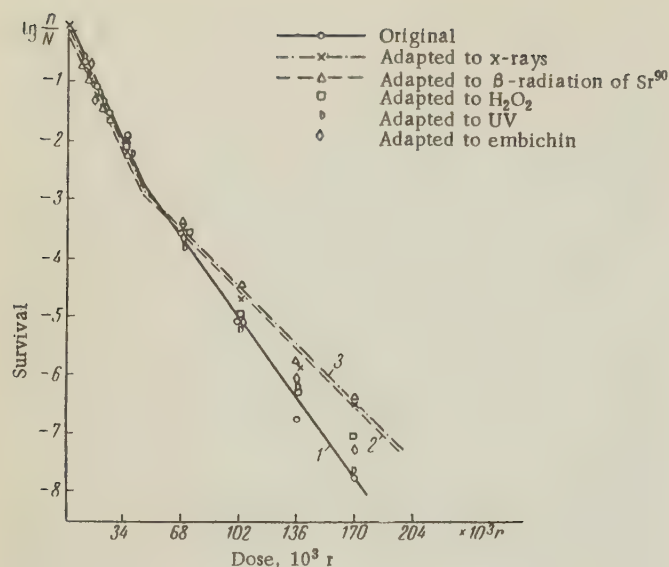


Fig. 2. Survival of *B. faecalis alcaligenes* strain 415 and of its variants when irradiated with x-rays. 1) Original; 2) adapted to β -radiation of Sr^{90} ; 3) adapted to x-rays.

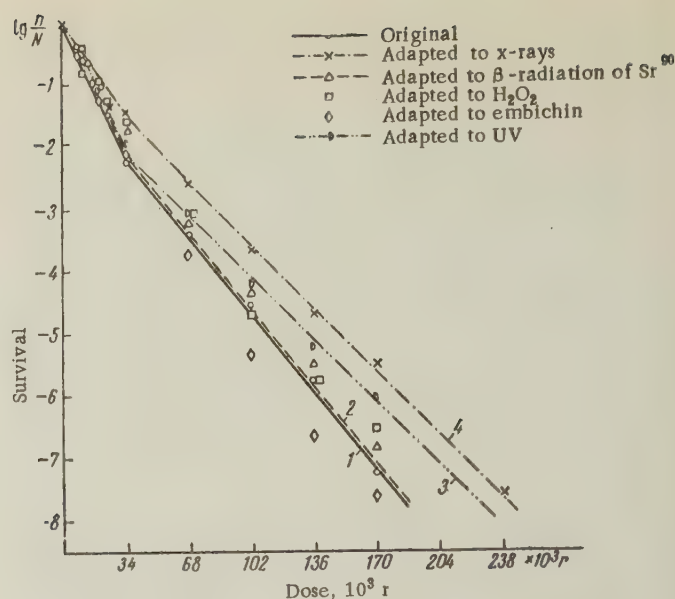


Fig. 3. Survival of *B. faecalis alcaligenes* strain M and of its variants when irradiated with x-rays. 1) Original; 2) adapted to β -radiation of Sr^{90} ; 3) adapted to uv; 4) adapted to x-rays.

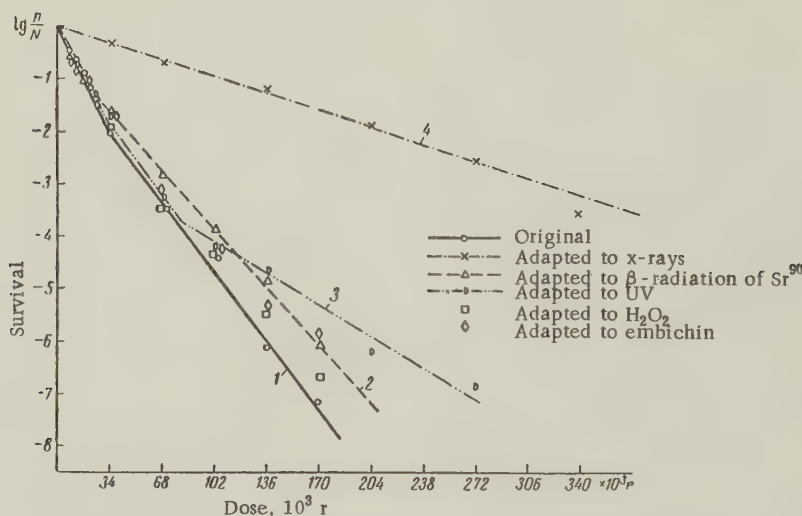


Fig. 4. Survival of *B. faecalis alcaligenes* strain 4 and of its variants when irradiated with x-rays. Designations same as in Fig. 3.

x-rays; therefore their survival curves are not specially discussed here.

In our experiments, adaptation to hydrogen peroxide and embichin, which increased the resistance of the cultures to these agents, did not lead to a parallel increase in their radioresistance. However, resistance to x-rays and ultraviolet irradiation was accompanied by increased resistance of these variants to the action of hydrogen peroxide. Thus, the bactericidal doses of hydrogen peroxide for variants of strain 4 which were resistant to x-rays and to ultraviolet increased 3.3 and 5.1 times, respectively. Resistance to hydrogen peroxide increased to a lesser extent (2.2 and 1.9 times) in variants of strain M which had been subjected to treatment with x-rays and ultraviolet irradiation.

In strain 4, the acquisition of resistance to the action of radiation and embichin also led to a considerable (46–56 times) reduction in sensitivity to streptomycin. Analogous treatment of strain M, on the contrary, increased the sensitivity of these variants to the antibiotic.

DISCUSSION

The survival curves for the x-ray irradiation of original cultures and certain adapted variants, represented on a semilogarithmic scale, are not expressed by absolutely straight lines, but are rather composed of several exponential curves. Gunter and Kohn (1956) obtained this type of survival curve for

E. coli treated with x-rays. The thought expressed by Gunter and Kohn concerning the unequal radiosensitivity of various cells in the population of such cultures can serve as a possible explanation of this. The cells in the population of variants which we obtained evidently became extremely heterogeneous with regard to radiosensitivity under the influence of some of the factors used and, along with the ordinary, sensitive individuals, highly resistant individuals also appeared in certain numbers. It is the presence of the latter in the population which possibly alters the course of the survival curve of cultures when large doses of radiation are used. With small doses, the survival of these variants may not vary from that of the original culture, possibly due to the radiosensitive cells existing in the population.

The selection of radioresistant cells considerably speeds up the process of obtaining variants highly resistant to radiation, the cells of which are more homogeneous in their behavior toward radiation (strain 4, adapted to x-rays).

In the effect of various types of ionizing radiations on microorganisms, along with points of similarity, there are definite differences which are possibly associated with the relative predominance of the direct or indirect mechanism of action of the ionizing radiation in inactivating different strains of microbes. Therefore, an increase in resistance to the effect of the β -radiation of Sr^{90} in our experiments did not always lead to increased resistance to x-rays.

Since we were not concerned with studying the question of the stimulatory effect of ionizing radiations on the processes of growth and metabolism in microorganisms, we did not observe this effect in our experiments; the doses of the β -radiation of Sr^{90} which we employed caused the retardation of cell division in growing cells of the microorganism. Only repeated or prolonged irradiation led to the appearance of variants having relatively rapid growth as compared with the original cultures. The relatively large magnitude of the total dosage (about 4700 rad) and high intensity (1.66 rad/minute) of the β -radiation of Sr^{90} in our experiments may be the explanation. It was shown by Sokurova (1956, 1957a, b) that stimulation of the processes of growth, metabolism, and cell division in root-nodule bacteria and azotobacter was manifested only in cases where radiations of low intensity and in certain total dosages were used. Increasing the dosage or intensity of radiation led to the inhibition of these processes.

In our cultures, adaptation to hydrogen peroxide was not accompanied by increased resistance to radiation. Variants which were resistant to x-rays, on the contrary, became more resistant to the action of hydrogen peroxide. Evidently, the peroxide mechanism is actually not entirely responsible for the biological effect of ionizing radiations, although it does have some significance (Shchepot'eva et al., 1959; Barron, 1955).

The nonspecific increase in resistance to streptomycin (in our experiments) or to penicillin in E. coli as described by Witkin (1947) in cultures resistant to

radiation depends, it seems to us, on the individual peculiarities of the experimental strains.

SUMMARY

1. Prolonged exposure of a culture of B. faecalis alcaligenes to hydrogen peroxide, embichin, and ultraviolet and ionizing radiation increases their resistance to these factors. However, the increase in resistance to ultraviolet, x-rays and β -radiation of Sr^{90} is not always observed, being dependent on the individual peculiarities of the experimental cultures and the irradiation procedure.

2. The increase in resistance of microorganisms to hydrogen peroxide is not accompanied by a change in sensitivity to ionizing radiation. On the contrary, microbe variants highly resistant to x-rays and ultraviolet radiation proved more resistant to hydrogen peroxide.

3. In some cultures, acquisition of resistance to ultraviolet rays results in increased radioresistance.

4. The increase in resistance of microorganisms to β -radiation of Sr^{90} is, as a rule, accompanied by an increase in resistance to x-rays, although such a parallelism in radioresistance to different kinds of radiation is not a strictly regular nor universal phenomenon.

5. Adaptation to embichin does not result in decreased radioresistance.

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THE INDUCER OF THE SECOND PHASE OF NITRIFICATION

IV. THE DEHYDROGENASE ACTIVITY OF A WASHED SUSPENSION OF NITROBACTER WINOGRADSKYI BUCH

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Nitrite is the only oxidizable substrate for *Nitrobacter*. Despite numerous attempts, it has not been possible to find another substance which could be oxidized by this strictly autotrophic organism.

In order to determine whether *Nitrobacter* actually has such an exclusive specificity, experiments were undertaken to study the oxidative capacity of washed suspensions of the organism.

A study of the dehydrogenase activity in Thunberg tubes with methylene blue proved to be a convenient method. This method is quite sensitive and permits the testing of the effects of a wide variety of substances.

METHODS

The *Nitrobacter* culture (Zavarzin, 1958) was grown in 5-liter flasks with bubbling of air. The cells were sedimented by centrifugation, were separated from the mineral sediment, washed, and suspended in cold phosphate buffer at pH 7.2. They were used for experiments the same day; otherwise, the activity decreased sharply, even when stored in the frozen state. Prior to centrifugation, the purity of the culture was checked by plating on meat-peptone or potato agar and by microscopic examination. Contaminated cultures were discarded.

For the experiment, 1 ml of suspension containing 1 billion cells/ml (determined nephelometrically) was placed in the bulb of the Thunberg tube. Into the tube were placed 0.5 ml of 1/20,000 methylene blue (0.156 μ M), 0.5 ml of substrate, usually in a concentration of 0.1 mM/ml, and 1 ml of 1/5 M phosphate buffer at pH 7.2. The tubes were kept under vacuum for five minutes from the start of vigorous gas evolution, and were then sealed and placed in a water bath at 36.0°. The temperature was equilibrated for 20 minutes; then, the contents of the bulb were tipped into the tube. The time of 90% decolorization of the methylene blue was determined by comparison with a standard. The experiment was terminated after 2 hours.

The experiments were set up in duplicate and were repeated three times for each substance, using different suspensions.

EXPERIMENTAL RESULTS

Suspensions of *Nitrobacter* cells from the end of the logarithmic or the beginning of the stationary phase

were able to decolorize methylene blue due to internal reserves. Various substances greatly affected the decolorization rate (Table 1).

It was found that the majority of substances reduced the rate of endogenous decolorization of methylene blue or stopped the process entirely. The following acids had particularly strong inhibitory effects: succinic, malic, citric, fumaric. The absence of decolorization with succinic acid could serve as a rather sensitive supplementary test for determining the purity of the cultures in these experiments.

Malonic acid, an inhibitor of the Krebs cycle, did not inhibit endogenous decolorization. Lactic and α -ketoglutaric acids even stimulated the process slightly. Carbohydrates, with the exception of ribose and acetaldehyde, proved to be highly toxic.

These are the results that might have been expected, because the majority of these substances have a negative effect on *Nitrobacter* in cultures. However, a number of organic substances increased the rate of methylene blue decolorization about twofold. The following proved to be such substances: pyruvic acid, benzaldehyde, xanthine, hypoxanthine (Table 2). The rest of the substances tested were more or less indifferent. The addition of nitrite did not increase the rate of methylene blue reduction.

Table 1. The Effect of Organic Substances on Methylene Blue Reduction by a Washed Suspension of *Nitrobacter*

Inhibit endogenous methylene blue reduction	Have no noticeable effect	Stimulate by less than 30%	Stimulate by more than 30%
Succinic acid	Formic acid	Lactic acid	Benzaldehyde
Malic acid	Oxalic acid	Ribose	
Fumaric acid	Acetic acid	α -ketoglutaric acid	Pyruvic acid
Citric acid	Ethanol	Malonic acid	Xanthine
Glucose	Methanol		Hypoxanthine
DPN			
Acetaldehyde			
Glycolic acid			
Phosphate esters of sugars			

Table 2. Decolorization of Methylene Blue by a Washed Suspension of *Nitrobacter* in the Presence of Some Organic Substances

Substance	H ₂ transferred in μ liters/hour per cell (parallel determinations)					
Sodium pyruvate	0.100	0.150	—	0.155	0.350	0.110
Benzaldehyde	0.070	0.140	0.180	0.175	0.405	0.115
Xanthine	—	—	0.140	0.160	0.280	0.860
Hypoxanthine	—	—	—	—	0.310	0.860
Control	0.028	0.090	0.090	0.00	0.190	0.070

Some of these substances may have served as electron donors for the anaerobic reduction of nitrate. Thus, in the presence of benzaldehyde, but not of acetaldehyde, the *Nitrobacter* suspension reduced nitrate to nitrite under anaerobic conditions. Under aerobic conditions, nitrite naturally did not accumulate.

When oxygen consumption was determined in the Warburg apparatus, it was found that the rate of endogenous oxygen consumption increased by about 1/3 when benzaldehyde, xanthine, and hypoxanthine were added, while the addition of nitrite increased consumption 100 times. These results are in agreement with the data of Bömeke (1939), who also found that calcium lactate stimulates endogenous respiration.

Thus, enzyme activity of the xanthine oxidase type was found in *Nitrobacter* suspensions using three electron acceptors—methylene blue, nitrate, and oxygen.

Not a single one of the substrates which stimulated methylene blue reduction could serve as an energy source for the growth of the *Nitrobacter* culture. None of the experiments on the cultivation of *Nitrobacter* on these substrates gave the slightest increment in the number of cells in comparison with the inoculum added.

The inhibition of the endogenous reduction of methylene blue by the components of the tricarboxylic acid cycle in the cell suspension makes the existence of this cycle as a dissimilatory mechanism unlikely in *Nitrobacter*. The results obtained cannot be attributed to cell permeability, since pyruvic acid, which is similar in structure, apparently penetrates the cell.

The discovery of enzyme activity of the xanthine oxidase type in *Nitrobacter* is in agreement with an earlier expressed hypothesis (Zavarzin, 1958) concerning the participation of a molybdoflavoprotein in

the nitrite-oxidizing system. The oxidation of aldehyde and purines can be attributed to a side reaction of this enzyme. At the same time, activity with the indicated substances serves as an indirect proof of its presence. Inasmuch as it was not possible to detect any signs of reproduction of *Nitrobacter* on pyruvic acid, benzaldehyde, xanthine, and hypoxanthine, it must be recognized that enzymatic activity with these substances has no physiological significance.

SUMMARY

1. A washed suspension of *Nitrobacter* can reduce methylene blue under anaerobic conditions.
2. The majority of the acids of the tricarboxylic cycle and glucose inhibit the endogenous decolorization of methylene blue by the *Nitrobacter* suspension, which does not agree with the possible participation of the tricarboxylic cycle in the metabolism of this organism.
3. Benzaldehyde, pyruvic acid, xanthine, and hypoxanthine increase the rate of methylene blue reduction about twofold.
4. Under anaerobic conditions in the presence of benzaldehyde, the *Nitrobacter* suspension reduces nitrate to nitrite.
5. The results obtained indicate the presence of an enzyme of the xanthine oxidase type in *Nitrobacter*.
6. *Nitrobacter* does not utilize benzaldehyde, pyruvic acid, or purines as substrates for growth.

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THE TRANSFORMATION OF PHOSPHORUS COMPOUNDS IN YEAST AT VARIOUS STAGES OF ALCOHOLIC FERMENTATION

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The dependence of the content and composition of phosphorus compounds in microorganisms on the conditions of their cultivation has been demonstrated by many investigators.

Investigations of the transformations of various forms of phosphorus in yeast have, as a rule, been carried out while culturing the cells under aerobic conditions (Wiame, 1949; Korchagin, 1954; Bukhovich and Belozerskii, 1958 and others). It was of definite interest to study changes in the various phosphates of yeast multiplying during anaerobic fermentation.

It is well known that the batch method of alcoholic fermentation is characterized by the fact that the conditions of vital activity of the yeast cells are drastically altered during a relatively short period of time. This circumstance makes it possible to follow the changes in phosphorus compounds in yeast at various stages of growth and reproduction. In this case, it is very important to establish the pattern of changes of acid-soluble and acid-insoluble phosphates during the more active phases in the life of the microorganism, namely, during the lag phase and during the period of logarithmic growth of the population. By varying the amount of yeast inoculum, it was possible to alter the duration of the lag phase and even to eliminate it.

In the present work, data are presented which characterize the transformation of acid-soluble and acid-insoluble phosphates in yeast at various phases of anaerobic fermentation.

METHODS

A natural nutrient medium prepared by cooking grain to a pulp in three parts of water at 145°, as is usually employed in industry, served as the substrate for the multiplication of yeast cells (*Saccharomyces cerevisiae* race XII) and for fermentation.

In order to prolong the lag phase or to eliminate it, seed yeast was used in various quantities. Fermentation was carried out first with a small amount of seed yeast (11-15 million cells per 1 ml of fermentable substrate), then with the optimal amount of cells (20-27 million per 1 ml), which corresponds to amounts of yeast cells ordinarily used in the alcohol industry, and finally, fermentation was carried out with a maximal amount of seed yeast (86.5 million per 1 ml).

The method of extracting acid-soluble phosphates was adopted from the works of Belozerskii and Kulaev (1957) and of Bukhovich and Belozerskii (1958). Phosphorus was determined by the Fiske-SubbaRow method with subsequent colorimetry using a photoelectric colorimeter.

Transformation of Phosphorus Compounds during Fermentation with Small Amounts of Seed Yeast.

As has already been noted above, with a small amount of seed yeast, the lag phase was prolonged and was characterized by retardation of growth and cell reproduction and by the absence of any outward manifestation of vital activity. At the same time, practically no assimilation of phosphorus from the nutrient medium occurred during the lag phase, nor was there any indication of the biosynthesis of organic compounds.

Therefore, it could be assumed that the distinguishing character of the lag phase must in some manner also become evident in the character of the transformation of phosphorus compounds. In actuality, it was found that in the stationary period of development of the yeast, the process of interconversion of the different forms of phosphorus in the cells occurred. With a clearly expressed and relatively long lag phase corresponding to the first three hours of fermentation, the phosphorus content of the acid-insoluble fraction increased in comparison with the seed yeast and reached its maximum in this time, equaling an average of 81% of the total phosphorus of the yeast (Fig. 1). At the same time, the amount of soluble phosphate constituted 11-14%. Only then, in the next three hours of fermentation, in connection with the end of the lag phase and the onset of the second intermediate growth period of the yeast, was an increase in the number of budding cells and a sharp change in the ratio of the various forms of phosphorus observed. The absolute quantity of phosphorus in the yeast rose, but the amounts of the various forms of phosphorus increased at different rates. Whereas the soluble phosphate content increased from 0.16 to 1.63 mg in three hours in yeast centrifuged out of 100 ml of fermentable substrate, i.e., increased 10.2 times and reached its maximum, the amount of acid-soluble phosphate increased only from 1.02 to 3.50 mg, i.e., only 3.4 times. In this case, the ratio of the indicated forms of phosphorus, expressed in percent of its total content in the

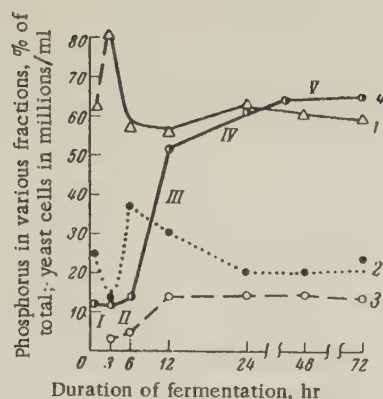


Fig. 1. The content of various phosphates in yeast. 1) Acid-insoluble phosphorus; 2) acid-soluble; 3) alcohol-soluble; 4) number of cells and their developmental phases (I-V).

yeast, constituted 1.6:1.0, i.e., there was an average of 34% soluble phosphate and 56% acid-insoluble phosphate.

After a 6-hour fermentation, corresponding to the period of logarithmic growth of the cells, acid-soluble and acid-insoluble phosphates increased at an equal rate; they showed a relative increase of 1.6–1.7 times in six hours. However, their absolute content in yeast centrifuged out of 100 ml of fermentable substrate was different and comprised 1.63 mg acid-soluble and 2.70 mg acid-insoluble in 12 hours (Table 1), or 31 and 56% of the total yeast phosphorus, respectively (Fig. 1).

The subsequent (fourth, or intermediate) period, which corresponded to the interval of from 12 to 24 hours, was characterized by retardation of the rate of cell multiplication and by sharp demarcation of the processes of synthesis of acid-soluble and acid-insoluble phosphorus compounds. The production of soluble phosphate ceased during this time and, to the contrary,

its content even decreased substantially and reached its minimum, corresponding to 1/5 of total yeast phosphorus. As for the acid-insoluble phosphorus, its content continued to rise and reached its maximum of 64% by the 24th hour.

Finally, in the fifth (or stationary) period, a decrease in the amount of phosphates was observed, this pertaining to acid-insoluble forms to a greater extent than to soluble phosphates. Apparently, yeast cells in the resting stage must necessarily contain at least a minimal amount of soluble phosphate, which represents a sort of "reserve metabolic fund" of the cell. It is well known that the assimilation of phosphorus from nutrient medium is associated with the presence of the necessary energy, which is released during anaerobic and aerobic respiration. In connection with this, it can be supposed that soluble phosphates and their intracellular transformation serve as an energy source for yeast cells in the lag phase.

The Transformation of Various Phosphates during Fermentation with an Optimal Amount of Seed Yeast.

In the present experiments, the lag phase was practically absent in the development of the yeast. For example, whereas in the first series of experiments no cell reproduction was observed during the first three hours, in the present experiments, the number of cells increased by 20 million, i.e., nearly doubled during the indicated time (Fig. 2). As a consequence of this, the content of acid-soluble phosphate was already maximal (51.8%), while the content of acid-insoluble phosphate was minimal (39.3%) in the first three hours of fermentation.

In the next period (from 6 to 12 hours), corresponding to the phase of logarithmic growth, the absolute quantity of acid-soluble phosphate still rose (from 1.11 to 2.32 mg in yeast from 100 ml of fermentable substrate), but relatively, it had already decreased and constituted 39% of the total phosphorus of the

Table 1. The Content of Various Forms of Phosphorus in Yeast at Different Stages of Fermentation (mg in the Biomass Centrifuged out of 100 ml of Fermentation Mixture)

Conditions of fermentation	Fermentation time, hr	Biomass dry wt.	Total	Alcohol-soluble	Acid-soluble	Acid-insoluble
With small amount of seed yeast	3	80.8	1.22	0.03	0.16	1.02
	6	106.3	2.76	0.15	1.03	1.58
	12	257.8	5.05	0.67	1.63	2.70
	24	541.2	5.41	0.80	1.41	3.50
	48	434.0	5.30	0.74	1.17	3.12
	72	393.9	2.97	0.40	0.77	1.80
With optimal amount of seed yeast	Initial	142.6	2.00	0.30	0.51	1.20
	3	145.7	2.18	0.23	1.11	0.91
	6	209.7	4.88	0.64	1.96	2.04
	12	440.8	6.10	1.11	2.32	2.69
	24	564.4	6.27	1.33	2.11	2.87
	48	416.3	4.87	0.73	1.57	2.57
	72	348.2	3.46	0.57	0.89	1.86
With maximal amount of seed yeast	Initial	492.0	6.80	0.40	3.87	2.52
	3	601.3	7.70	0.44	5.20	2.07
	6	808.0	9.11	0.62	6.87	2.61
	12	953.2	10.75	0.83	7.00	2.92
	24	851.6	9.64	1.05	2.50	6.09
	48	727.6	9.00	1.25	2.48	5.67
	72	640.0	7.90	0.85	1.75	5.30

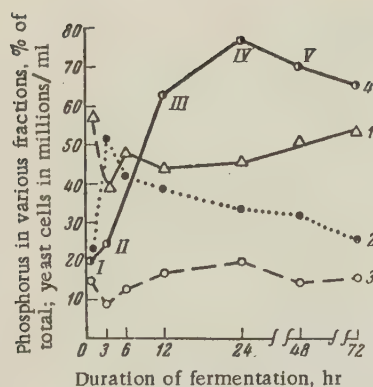


Fig. 2. Content of alcohol-soluble, acid-soluble, and acid-insoluble phosphorus in yeast. Designations same as in Fig. 1.

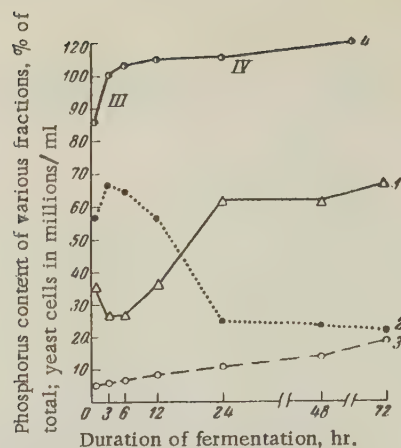


Fig. 3. Content of alcohol-soluble, acid-soluble, and acid-insoluble phosphorus in yeast. Designations same as in Fig. 1.

Table 2. The Content of Total and Acid-Soluble Phosphorus in Yeast at Various Stages of Alcoholic Fermentation

Analytical indicators		Duration of fermentation, hr					
		3	6	12	24	48	72
Total phosphorus	% of dry weight	1.30	2.15	1.94	1.51	1.60	1.48
	mg per 10^{10} cells	9.53	10.50	9.21	8.32	7.62	4.87
Acid-soluble phosphorus	% of dry weight	0.58	1.01	0.71	0.37	0.31	0.30
	mg per 10^{10} cells	5.62	5.21	4.78	2.45	2.09	1.39

yeast (instead of 51.8%) or 0.57% of dry weight of biomass instead of 0.75%.

The amount of phosphorus in the acid-insoluble fraction, on the other hand, increased constantly, and was considerably higher during the entire subsequent period of fermentation than at the start of the experiment (Table 1).

When comparing the results of the two series of experiments described above, it should be noted that in the experiments with a clearly pronounced lag phase, the greatest content of soluble phosphate in the yeast (and the least insoluble) was observed only after six hours of fermentation, while under conditions where the lag phase was absent, it already reached a maximum value after a 3-hour fermentation.

At the same time, the amount of soluble phosphate exceeded the content of acid-insoluble phosphorus in experiments without a lag phase, while in the presence of a lag phase, no such relationship was observed.

The Transformation of Various Phosphates during Fermentation with a Maximal Amount of Seed Yeast.

When a large amount of seed yeast was added to the fermentable substrate, the lag phase was eliminated from the development of the yeast cells.

Whereas in the fermentation with minimal amounts of seed yeast, a further decrease in acid-soluble phosphate was observed during the lag phase as compared

with the initial yeast, this process was eliminated in fermentations with a maximal amount of seed yeast. The active yeast already had a very high content of soluble phosphorus (67% of total phosphorus) from the first hour of fermentation; moreover, it remained essentially unchanged during the next three hours as well. At the end of fermentation, however, there was 22% acid-soluble phosphorus instead of 67%, and, on the contrary, 63% insoluble phosphorus instead of 26.9% (Fig. 3).

Consequently, in the course of alcoholic fermentation, the content of acid-soluble phosphates in yeast changes drastically and frequently, depending on the physiological state of the yeast: At the beginning and end of fermentation, yeast contains the least and approximately equal amounts (20–25%) of acid-soluble phosphates; after 3–6 hours of fermentation, a maximal content of soluble phosphates is found in the yeast; twice—after 1–2 hours and after 8 hours—an amount equal to acid-insoluble phosphates is found. The yeast had a relatively constant phosphorus composition at the end of fermentation regardless of the conditions of multiplication.

Of the total amount of yeast phosphorus, acid-insoluble phosphates accounted for an average of 60%, acid-soluble for 23%, and alcohol-soluble for 17%. The most active cells contained the maximal amount of

Table 3. The Content of Various Acid-Soluble Forms of Phosphorus in Yeast at Various Stages of Fermentation

Time of fermentation, hr	Orthophosphate			Labile phosphorus			Stable phosphorus		
	mg*	% of dry weight	% of total yeast P	mg*	% of dry weight	% of total yeast P	mg*	% of dry weight	% of total yeast P
3	0.41	0.22	14.4	0.34	0.19	8.2	—	—	—
6	0.74	0.35	9.8	0.66	0.31	8.5	0.36	0.20	6.9
12	0.64	0.22	9.9	0.82	0.19	7.3	0.83	0.23	12.5
24	0.54	0.10	8.9	0.56	0.10	6.8	0.78	0.16	14.7
48	0.34	0.08	6.9	0.61	0.14	8.8	0.65	0.16	14.3
72	0.24	0.09	6.7	0.39	0.11	6.1	0.33	0.11	11.3

* As mg in yeast centrifuged out of 100 ml of fermentation mixture.

Table 4. The Content of Acid-Soluble Polyphosphates in Yeast

Analytical indicators	Duration of fermentation, hr					
	3	6	12	24	48	72
Polyphosphate phosphorus in 10^{10} cells, mg	0.38	0.40	0.21	0.06	0.03	0.03
Yeast cells, 10^6 /ml	13.0	24.4	64.5	63.3	64.1	61.2
Budding cells, %	18.0	30.0	11.3	7.0	5.3	4.4

acid-soluble phosphorus and the minimal amount of acid-insoluble phosphorus. The start of intensive cell multiplication was evidently associated with a maximal accumulation of the soluble forms of phosphorus in them.

For example, during the first 3–6 hours of fermentation, the yeast contained maximal amounts of both total and acid-soluble phosphorus calculated as percent of dry weight of biomass or per 10 billion cells (Table 2).

The development of yeast during the lag phase is characterized by increased enzymatic activity and intracellular transformation of soluble phosphorus compounds including all three forms of phosphates, namely: orthophosphate, labile and stable phosphorus (Table 3).

In addition to this, orthophosphate is consumed in the largest amount, followed by labile phosphorus; the content of stable phosphorus in the yeast remains essentially unchanged during nearly the entire period of fermentation.

Of the soluble forms of phosphorus, the polyphosphates are of paramount significance; these are substances which evidently stimulate the budding of cells, since, when yeast ceases to multiply, the amount of soluble polyphosphates in it decreases to a minimum. For example, whereas during the first 3–6 hours of fermentation, there was 0.38–0.40 mg of polyphosphate phosphorus per 10 billion yeast cells, after a one-day and three-day fermentation, its content was, corre-

spondingly, 0.06–0.003 mg, i.e., it was many times less (Table 4).

SUMMARY

1. Yeast cells show quantitative and qualitative differences in the composition of phosphorus compounds at various stages of development.

2. Intracellular transformation of phosphorus compounds in yeasts is noted in the lag phase, i.e., when reproduction and phosphorus consumption have not yet begun.

3. During the subsequent period of yeast development linked with an increase in the rate of cell reproduction the amount of soluble phosphates increases.

Intense budding and cell reproduction are linked with the highest content of soluble phosphorus compounds, which consist of both labile and stable phosphorus as well as polyphosphates.

4. During the logarithmic growth phase the increase in acid-soluble and acid-insoluble phosphates in yeasts proceeds at a relatively similar rate, although their ratio is changed drastically. Of the total yeast phosphorus, the acid-soluble forms amount to about 30%, the acid-insoluble ones to 56%, and alcohol-soluble ones to 14%.

5. During the subsequent stages of yeast development, while no apparent consumption of extraneous phosphorus takes place, further intracellular transformation of acid-soluble phosphates into acid-insoluble phosphorus compounds is to be noted, as in the

lag phase. The content of soluble phosphates drops to a minimum corresponding to 1/5 of total yeast phosphorus.

6. The direct correlation between yeast reproduction and the maximum content of acid-soluble polyphosphates, as well as further, almost complete consumption of these compounds, points to the conclusion that polyphosphates may be regarded as compounds which determine the initiation and rate of cell budding.

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THE SPLITTING OF PECTIN COMPOUNDS BY ENZYMES OF MICROORGANISMS AND THE CHEMICAL NATURE OF THE END-PRODUCTS

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The effects of enzymes on the pectin compounds of plants are numerous and varied (Barinova, 1946; Gaponenkov, 1954; Novotel'nov and Deeva, 1949; Oparin, 1947).

The processes of flax retting, changes in fruits and vegetables during storage, the clarification of fruit and berry juices, production of wines, and the decomposition of vegetable residues in the soil are to a greater or lesser extent associated with the transformations which pectin compounds of plants undergo due to the effects of the enzymes of microorganisms (Barinova, 1946; Rubin, 1945; Rudakov, 1949).

An interconnection between the ability of microorganisms to affect plant tissues and the presence in them of pectin-decomposing enzymes has been noted in the literature (Shishelova, 1948).

The splitting of the pectin compounds of plants by enzymes can proceed through a number of intermediate stages as far as the formation of free d-galacturonic acid (Novotel'nov and Deeva, 1949; Luh and Phaff, 1954; Demain and Phaff, 1954).

The mechanism of the enzymatic splitting of pectin compounds has not yet been determined and, furthermore, the chemical nature of the end-products has not been definitely established. The solution of these questions is of great theoretical and industrial significance and is attracting the attention of investigators; therefore, the present work is devoted to the clarification of some of the questions in this complex problem.

EXPERIMENTAL METHODS

Pectin obtained from sunflower calathides and pectinase produced by the mold Aspergillus niger were used in the investigations.

Pectin was obtained from sunflower calathides by extraction with 0.4% HCl (pH 2.3-2.6) for one hour at 80°. After filtration, the pectin was precipitated out of the extract with an aqueous solution of $AlCl_3$ (1:4) with the addition of ammonia in order to alkalize to pH 3.4-3.8.

The pectin precipitate obtained was dried, broken up, washed first with 50% aqueous alcohol, then with a solution of 55% alcohol and 0.3-0.4% ammonia, after which it was dried and pulverized.

The pectin obtained had the following properties (in %): moisture-14.7; galacturonic acid-72.75; methoxyl groups-5.95; total ash-2.00; aluminum-0.355.

The pectinase preparation was obtained by means of growing A. niger on autoclaved, previously crushed sunflower calathides. After 42 hours of growth at 28°, the sprouted mass was ground with quartz sand, flooded with water in a ratio of 1:1.5, and subjected to autolysis at 42° for 16 hours.

Three volumes of alcohol were added to the filtered liquid. The precipitate formed was filtered off and washed with alcohol and ether, dried over calcium chloride, and pulverized.

Enzyme activity was determined by the change in viscosity of pectin hydrosols in citrate buffer at pH 4.2, a temperature of 40°, and an enzyme concentration of 0.2 g per 100 ml of solution.

In order to determine the effect of methoxyl groups and ash elements included in the composition of pectin on the process of enzymatic splitting of pectin, demethoxylated and de-ashed preparations were obtained. Ash was removed from pectin by passing pectin hydrosol through a column of KU-1 cation exchange resin. The ash content of the preparation obtained was 0.45%.

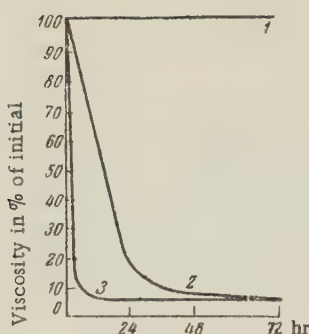
Demethoxylation was carried out by treatment with 4% NaOH, and de-ashing—by passing the solution through a column of cation-exchange resin. The preparation obtained contained 0.8% methoxyl groups and 0.45% ash.

When experiments were done to determine the activity of the pectinase extracted from the A. niger culture, it was noticed that the cloudy, viscous hydrosol of sunflower pectin became transparent and fluid.

These observations of outward appearance suggest that pectin molecules are broken down very rapidly after the addition of pectinase. Proceeding from the current views of the structure of the pectin molecule, it can also be assumed that the breakdown of the molecule occurs at the sites of glucoside linkages between residues of d-galacturonic acid.

Thus, viscosimetric determinations can give us some idea of the extent of breakdown of the pectin molecule as related to the conditions under which the experiment was performed.

We conducted a series of experiments on the enzymatic splitting of pectin at 40° for 1-72 hours. Viscosity



Changes in viscosity of 2% pectin hydrosol as a function of time at 40°. 1) Pectin hydrosol; 2) pectin hydrosol with enzyme; 3) pectin hydrosol with enzyme in citrate buffer.

Table 1. Extent of Breakdown of Pectin at 40° in Percent

System	Extent of breakdown of pectin molecule in % after					
	1 hr	10 hr	24 hr	48 hr	72 hr	96 hr
2.0% pectin hydrosol (control)	No breakdown occurs					
2.0% pectin hydrosol + 0.2% pectinase	—	37.05	85.0	95.9	97.9	97.9
2.0% pectin hydrosol in citrate buffer + 0.2% pectinase	91.1	—	98.4	98.4	—	98.4

was determined in an Ostwald viscosimeter. Changes in viscosity were expressed as percent of initial viscosity. The following systems were used for this purpose: 1) 2.0% pectin hydrosol without enzyme; 2) 2.0% pectin hydrosol + 0.2% pectinase, pH 3.4; 3) 2% hydrosol + 0.2% pectinase at pH 4.2 (in citrate buffer). The data obtained are represented in the figure.

We next attempted to calculate the extent of breakdown of the pectin molecule in percent according to the formula (Karakolev et al., 1956):

$$A = \frac{t_a - t}{t_a - t_0} \cdot 100,$$

where t = time of flow of the pectin hydrosol treated with enzyme, t_a = time of flow of the original pectin hydrosol, and t_0 = time of flow of solvent. The data obtained are given in Table 1.

As seen from the figure and from Table 1, the sharpest drop in viscosity and the greatest disruption of the pectin molecule by pectinase occurred in the initial period at pH 4.2 in citrate buffer, and more slowly in the subsequent periods of fermentation. These facts indicate the complexity of the process of the enzymatic splitting of the pectin molecule.

The viscosimetric determination of the extent of breakdown of the molecule does not show us how completely the pectin molecule is hydrolyzed to the formation of d-galacturonic acid.

We therefore conducted a series of experiments to determine the mechanism of the enzymatic splitting

Table 2. Yield of Barium Salt Fractions in Percent

Alcohol concentration during precipitation, %	Experimental variants			
	1	2	3	4
25	50.8	14.44	10.65	10.15
40	32.45	29.40	18.50	29.15
50	8.35	21.52	21.05	20.7
90	8.30	34.64	49.80	40.0

of the pectin molecule as a function of the chemical nature of the initial products, the temperature, and the pH value, as well as by isolating and identifying the end products.

The enzymatic splitting of pectin compounds by *A. niger* pectinase over a period of one month was set up in the following variants: 1) pectin hydrosol at pH 3.4; 2) a hydrosol of demethoxylated and de-ashed pectin at pH 2.55, incubated at 40°; 3) hydrosol of demethoxylated and de-ashed pectin at pH 4.7, incubated at 20–22°; 4) hydrosol of de-ashed pectin at pH 4.5, incubated at 20–22°.

When fermentation was finished, the solutions were heated to 55–60°, Congo red indicator was added, and BaCO_3 was added (with frequent stirring) until the appearance of a stable red color. Under these conditions, the end-products of hydrolysis, having free carboxyl groups in their composition, formed water-soluble barium salts. After 24 hours of settling, the precipitate was filtered off. The filtrates were evaporated to small volumes (40 ml) under vacuum.

The barium salts were fractionated with different concentrations of alcohol: 25%, 40%, 50%, and 90%. The precipitates obtained were filtered off, washed with alcohol, dried first over sulfuric acid, and then in a drying cabinet at 105° to constant weight. The yield of barium salts in each fraction was calculated in percent of the total yield of salt (Table 2).

The barium salt fractions were then converted to free acids by passing their aqueous solutions through a column of KU-1 cation-exchange resin. The solutions obtained were evaporated, first under vacuum to a small volume, and then in a porcelain dish to the consistency of a thick syrup, and 96% alcohol was added. Precipitates formed under these conditions. The precipitates were filtered out, dried over sulfuric acid, and then to constant weight in a drying cabinet.

The resulting fractions of end-products of the enzymatic splitting of pectin compounds were characterized by the carrying out of both qualitative and quantitative reactions.

All fractions, without exception, showed reducing capacity: They reduced Fehling's solution, which indicates the presence of the aldehyde group in them.

The quantitative determination of aldehyde groups was carried out according to the Willstätter-Schudel method, and molecular weight was calculated on the basis of this (method of determining end-groups).

The determinations of molecular weights showed that the isolated fractions of end-products of the splitting of the pectin molecule by *A. niger* pectinase con

tained compounds with molecular weights of 1786, 714, 686, 550, 261, and 214.

Titration of the fractions with 0.1 N NaOH showed that each link in the chain contained one carboxyl group. The fractions with molecular weights of 261 and 214 formed brick-red precipitates with basic lead acetate.

When oxidized with nitric acid, mucic acid with a melting point of 200° was obtained, which gave an intense yellow color with ferric chloride.

All this speaks for the fact that poly-, tetra-, tri-, di-, and monogalacturonic acids are formed during the splitting of pectin by pectinase.

The barium salt fraction precipitated with 25% alcohol consisted chiefly of polygalacturonic acids (X coefficient greater than 4); the fraction precipitated by 40% alcohol consisted of tetragalacturonic acid; the fraction precipitated by 50% alcohol—of a mixture of tri- and digalacturonic acids, and the fraction precipitated with 90% alcohol—of monogalacturonic acid.

Examining the data given in Table 2, it can be said that the most complete splitting of the pectin molecule by *A. niger* pectinase occurs at pH 4.5–4.7 when it is demethoxylated and de-ashed, i.e., when carboxyl groups are released in the pectin molecule.

The carboxyl groups released apparently promote the more rapid and complete splitting of glucoside bonds in the chain of the pectin molecule.

Ash elements and methoxyl groups included in the composition of the pectin molecule make it more resistant to the action of enzymes.

These facts are of interest and help to give a more thorough knowledge of the process of enzymatic splitting of pectin compounds in plants.

SUMMARY

1. The splitting of pectin isolated from sunflower calathides by the pectinase of *A. niger* results in the formation of poly-, tetra-, tri-, di-, and monogalacturonic acids.

2. Elimination from the pectin molecule of methoxyl groups and of ash elements results in the most rapid and complete splitting of pectin to its end-products up to the formation of d-galacturonic acid.

Free carboxyl groups favor the rupture of glucoside bonds in the chain of the pectin molecule.

3. The highest activity of the pectinase of *A. niger* is noted at a pH of 4.5–4.7.

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TRANSFORMATION IN MICROBES INDUCED BY FILTRATES PASSED THROUGH BACTERIAL FILTERS

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There can be different approaches to the problem of transformation in microorganisms. For general microbiology in particular, the clarification of the possibility of transformation under conditions of symbiotic cultures is of decided interest. It is well known that when two cultures are grown together, hereditary changes in the properties of one or of both cultures are sometimes observed. Such variability is usually explained by the effect of the products of vital activity of one culture on the other. Meanwhile, it is quite obvious that even in relatively young cultures, autolysis and cell destruction is observed, adding to the medium cellular contents, including DNA specific for the given species or strain. In one of the preceding communications (Imshenetskii et al., 1959), we pointed out that it is sufficient to have present in the medium $1 \mu\text{g/ml}$ of DNA which had been isolated from the cells of a staphylococcus variant resistant to streptomycin in order to transfer this resistance to the original sensitive staphylococcus culture growing on this medium. Quite naturally, not only such, but even larger quantities of DNA are given off into the culture during the death and disruption of cells which is so frequently observed when culturing microbes. It would be very complicated to conduct a direct experiment to determine the significance of transformation in variability which is due to symbiosis, because in mixed culture, the cells introduced — the "DNA donors" — are indistinguishable from cells with similar properties arising as the result of transformation. This made it necessary to separate the donor and recipient cultures with a semipermeable membrane and to determine the possibility of transformation under these conditions. At the same time, these investigations may show to what extent substances causing transformation are capable of passing through such membranes.

EXPERIMENTAL METHODS

The investigation was carried out with a culture of *Staphylococcus aureus* 209, as well as with a culture of the same species but adapted to streptomycin by means of numerous passages on media containing constantly increasing concentrations of this antibiotic. The streptomycin-resistant culture was grown on meat-peptone agar (MPA) containing 40,000 units of streptomycin per 1 ml of medium. The original staphylococcus culture did not grow when inoculated on MPA slants

containing the same amount of streptomycin. However, when very large numbers of cells were plated on solid medium with the antibiotic, colonies of the resistant form grew very infrequently. It was established by earlier investigations that the frequency of the formation of resistant variants in the original culture was $1/10^9$. In performing the experiments the following scheme was followed. Two hundred ml of an 8-hour broth culture of the original (nonresistant) form of the staphylococcus was placed in a 300-ml Erlenmeyer flask. A glass tube on the end of which a collodion bag was fastened was put through the cotton stopper of the flask. Seven ml of a 4-day culture of the streptomycin-resistant staphylococcus strain was placed in the bag. Preliminary cultivation of this strain was carried out at 37° , on meat-peptone broth (MPB) containing 40,000 units of streptomycin per 1 ml. In this manner, the cultures of the resistant and nonresistant staphylococcus were separated by a semipermeable collodion membrane. The flasks were kept at 37° for 24 hours, and then the cells of the original sensitive staphylococcus cultures in the flasks were plated on MPA containing 40,000 units of streptomycin per 1 ml of medium. The inoculated plates were placed in an incubator, and, after 2-3 days, the number of colonies growing on the streptomycin-containing medium was counted.

EXPERIMENTAL RESULTS

Experiments with Collodion Bags

In carrying out the experiments for which the methods were given above, control experiments were of great significance. Therefore, various controls were set up simultaneously with the experiments.

First control. First of all, the possibility that the cells of the resistant form of the staphylococcus might pass through to the original culture of this species in the flask had to be eliminated. In connection with the detection of this possibility, in the first control, a broth culture of the resistant staphylococcus was placed in the collodion bag, while sterile broth was placed in the flask. There was not a single instance of the staphylococcus cells penetrating to the broth and growing in it. Consequently, the collodion membrane was impermeable to cells. This can not be said of cellophane, since we became convinced that staphylococci pass into the MPB through cellophane bags, apparently due to the development of cracks.

Second control. It is possible to suppose that sterile MPB was not entirely adequate for the growth of the staphylococcus cells which could have passed through the collodion bag into the MPB. Therefore, in the second control, which was essentially the same as the first, a culture of *Sarcina flava* was used instead of the MPB. In this case as well, the streptomycin-resistant staphylococcus did not pass through the collodion membrane and did not grow with the sarcina.

Third control. This was carried out for the purpose of demonstrating that the integrity of the collodion bags was not disturbed during the time of the experiment. For this purpose, after the end of the experiment, the contents of the bag (i.e., the resistant staphylococcus culture) was poured out and the bags were rinsed and then boiled for 20 minutes. Then, a broth culture of the resistant staphylococcus was again placed in the bags, and they were immersed in flasks of sterile MPB. The flasks were kept for 72 hours. The broth in them always remained sterile, and this proved that the integrity of the collodion bags was not impaired during the time of the experiment.

Fourth control. In connection with the fact that the resistant staphylococcus was grown on medium containing streptomycin, the supposition arose that the streptomycin present in the culture of the resistant staphylococcus penetrates through the membrane and, getting into the original culture, causes the appearance of resistant forms and at the same time brings about their selection. In carrying out the fourth control, an 8-hour broth culture of the original (nonresistant) form of the staphylococcus was placed in the flasks, while MPB containing 40,000 units of streptomycin per 1 ml was placed in the collodion bag. After incubating the flasks, the original staphylococcus culture from the flasks was plated on MPA containing 40,000 units of streptomycin per 1 ml. In these inoculations, no colonies of the resistant form appeared on the plates.

Thus, the controls carried out were convincing evidence of the fact that cells of the resistant staphylococcus never penetrated through the walls of the collodion bag and that consequently, the appearance of resistant forms in the original culture cannot be explained by contamination with the cells of the resistant culture. Furthermore, the controls refuted the possibility of the appearance of resistant cells through the effect of streptomycin present in the culture fluid of the culture which in the given experiments served as the "donor" of the substances causing transformations.

As for the experiments themselves, they provided a completely clear and definite answer to the question posed at the beginning of this paper. In the first series of experiments, colonies of the resistant form grew up when the original staphylococcus culture was plated on medium containing streptomycin. The frequency of the appearance of resistant variants was $1/0.17 \cdot 10^3$. In the second series of experiments, analogous data were obtained when the contents of 11 flasks were examined, and the frequency of appearance of the resistant forms was $1/0.2 \cdot 10^4$. Consequently, the substances contained in the 4-day culture of the resistant

staphylococcus diffused through the wall of the bag and caused transformation. It is interesting that the frequency of appearance of resistant mutations under these experimental conditions was no lower than when cells of the nonresistant culture were treated with various amounts of DNA isolated from the cells of the resistant variant (Imshenetskii et al., 1959).

The results obtained decidedly confirmed the existence in the culture fluid of substances capable of causing transformation. However, in the live culture of the resistant staphylococcus variant, the amount of such substances was incomparably lower than in cell-free extracts obtained by cell disruption of the streptomycin-resistant form of staphylococcus. The method for obtaining such preparations has been described in another of our articles (Imshenetskii and Perova, 1960). In connection with this, the frequency of production of resistant forms through the effect of the live culture had to be compared with that resulting from the parallel action of cell-free extract obtained from the cells of the resistant form. The scheme of the experiments was the same as before. A four-day culture of the resistant staphylococcus was placed in some collodion bags, while cell-free extract was placed in other bags. The results of these experiments are given in Table 1.

Table 1. Transformation Induced by a Cell-Free Extract and by a Live Culture of Staphylococcus

Flask No.	Frequency of production of resistant variants	
	Live culture	Cell-free extract
1	$1/0.8 \cdot 10^7$	$1/0.4 \cdot 10^7$
2	$1/0.2 \cdot 10^5$	$1/0.7 \cdot 10^5$

These results permit us to make the following conclusions: 1) both the cell-free extract and the live culture induce transformation; 2) the number of resistant mutations appearing is approximately the same in the experiments with the live culture as in the experiments with cell-free extract.

The circumstance that the live culture of the resistant staphylococcus gave the same number of resistant variants as the cell-free extract was somewhat unexpected. Theoretically, the reverse should have been expected, since cell-free extract apparently contains considerably more DNA than the culture fluid from a live culture. The live, reproducing culture may possibly contain less altered and consequently, more active DNA than the cell-free extract.

Experiments with Bacterial Filters

The passage of the substances causing transformation through the semipermeable membrane was of interest. It is commonly thought that transformation is brought about by high-polymer DNA, and that when it is depolymerized, its transforming capacity is sharply reduced. In our previous investigations, data were obtained which confirmed the correctness of this point of view, since DNA which was stored in the laboratory and which was consequently depolymerized showed

Table 2. Transformation in Staphylococcus Caused by Filtrates Obtained with the Aid of Bacterial Filters

Cell-free extract	Number of cells for which there was one mutation producing a resistant variant					
	Experiment No.					
	1	2	3	4	5	6
Filtered through asbestos filter, diluted 10x	$0.3 \cdot 10^5$	$0.3 \cdot 10^9$	$0.16 \cdot 10^5$		No growth	$0.46 \cdot 10^4$
Same, but diluted 2x	No growth	$0.3 \cdot 10^9$	No growth	No growth	" "	$0.19 \cdot 10^6$
Filtered through L ₃ candle, diluted 10x	$0.12 \cdot 10^9$	$0.12 \cdot 10^9$	$0.4 \cdot 10^5$	$0.3 \cdot 10^4$	" "	$0.77 \cdot 10^8$
Same, but diluted 2x	$0.5 \cdot 10^5$	$0.12 \cdot 10^9$	$0.4 \cdot 10^5$	$0.4 \cdot 10^6$	" "	$0.36 \cdot 10^1$
Unfiltered, diluted 10x	$0.1 \cdot 10^5$		$0.1 \cdot 10^3$	—	" "	$0.11 \cdot 10^4$
Control (number of resistant variants in original culture)	No resistant variants in $3.1 \cdot 10^8$ cells	$0.1 \cdot 10^9$		No resistant variants in $5.38 \cdot 10^8$ cells	" "	—

markedly reduced transforming activity. Moreover, treatment of the DNA with the enzyme desoxyribonuclease inactivated DNA to a considerable extent. The results described above suggested that high-polymer DNA passed through the semipermeable membrane, and it was therefore desirable to determine the possibility of the passage of DNA through fine-pore bacterial filters. For this purpose, one part of the cell-free extract obtained from the cells of the resistant staphylococcus form was filtered through an L₃ Chamberland candle, while a second part was filtered through an SF asbestos filter. A third part of the extract was not filtered at all. Then, each portion of the filtered extract was diluted two and ten times, and was added to the nutrient medium in which the original strain of staphylococcus was growing. In the controls, the same dilutions of unfiltered extract were added. Later, the frequency of appearance of transformed streptomycin-resistant variants was established by plating on streptomycin-containing agar.

The data of these experiments, given in Table 2, enable one to make the following conclusions: 1) when the filtrate passes through such relatively fine-pore filters as the L₃ candle and the SF asbestos filter, its ability to cause transformation is preserved; 2) irrespective of the character of the filtrate, there was no transformation in two of six experiments (experiments 2 and 5); 3) when the filtrate obtained with the aid of the L₃ candle was diluted two times, it was considerably more active than when it was diluted ten times; 4) cell-free extract filtered through an SF filter caused transformation when it was diluted ten times, but when it was diluted two times, a positive result was obtained in only one experiment; 5) filtration decreases the activity of the extract; more intensive transformation occurs when unfiltered extract is used.

Having analyzed the data in the table, it must be added that despite occasional unsuccessful experiments, transformation by means of filtrates was obtained in 15 experiments. At the same time, the number of resistant forms arising was two to three orders higher than the number of similar variants arising in the original streptomycin-sensitive staphylococcus culture. Consequently, there is no doubt that resistant forms can be obtained under these experimental conditions.

According to current concepts, the molecular weight of DNA fluctuates from 500 thousand to 100 million. Naturally, with such molecule size, as well as its filamentous structure, it is difficult for the high-polymer undisaggregated DNA to pass through the bacterial filter. It was shown above that during filtration, the transforming activity of cell-free extracts decreases in comparison with unfiltered extracts, and it is quite probable that this is due to the retention of DNA during filtration. However, such retention is relatively small, and this is confirmed by the positive results of the experiments on transformation. The question of to what extent the DNA contained in the cell-free extracts undergoes disaggregation requires additional investigation. The possibility that it is partially depolymerized in the process of preparation of the cell-free extracts, filtration, and performance of the experiment has not been excluded. In this case, it may be assumed that not only the high-polymer DNA, but DNA having a relatively low molecular weight as well, has a transforming effect.

SUMMARY

1. The symbiosis of microorganisms as a factor in variability has been known for a long time. Possible reasons for this variability may be not only the effect on the cells of the metabolic products accumulating in the culture, but also the transforming activity of DNA given off into the culture fluid during the disruption of the cells.

2. In order to check the hypothesis given above, streptomycin-resistant staphylococcus was cultured in collodion bags immersed in the broth culture of the original sensitive staphylococcus. Substances diffusing through the collodion membrane caused transformation, i.e., the appearance of streptomycin-resistant forms.

3. Transformation in staphylococcus can be brought about by extracts obtained from the cells of the resistant staphylococcus and filtered through an L₃ Chamberland candle or an SF asbestos filter. The transforming activity of the extracts decreased somewhat during filtration, but there was quite a considerable number of resistant staphylococcus forms arising.

4. The preservation of transforming activity during the filtration of extracts through relatively finely pored filters suggests that transformation can be brought about by other than just highly polymerized DNA of high molecular weight.

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THE EFFECT OF SOME RADIATION-PROTECTING COMPOUNDS ON THE MUTATION RATE OF AN UNSTABLE STRAIN OF BACTERIUM PRODIGIOSUM

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It is well known that the irradiation of cells and the effect of chemical mutagenic factors result in similar genetic effects.

Together with the direct effect of irradiation on unique cellular structures, compounds which possess mutagenic activity are formed after irradiation. It has also been hypothesized that natural spontaneous mutation is also caused by the radiomimetic properties of several metabolites (Wagner and Mitchell, 1958). It has been shown that radiation-protective compounds prevent the appearance of inheritable changes (Riley, 1955, 1957). We therefore considered it interesting to study the effects of several radio-protective compounds on natural mutation.

In the majority of organisms it is difficult to decrease the rate of mutation which is always occurring spontaneously at a slow rate. Rare instances are encountered, such as the so-called unstable inheritance, in which the frequency of mutation of some characteristic increases many times (Rizhkov, 1939). Evidently, several kinds of bacterial dissociation can be related to this category of change, such as the dissociation of pigment formation in *Bacterium prodigiosum* (Labrum and Bunting, 1953; Kaplan, 1948, 1952, 1953).

We have used this microorganism as a model for studying the effect of radioprotective compounds on natural variability.

METHODS

The stock strain B₁ of *B. prodigiosum* was obtained by us from the Department of Microbiology of the First Moscow Medical Institute; on agar with 1% glucose, colonies consisting of light and dark sections were formed (Figures 1 and 2). In transferring bacteria from different sections of a colony, two types grew out: dark colonies with one or two narrow light sections and light colonies with several broad dark sections. There was always a quantitative relation between colonies of both types transferred from either the dark or light section (Fig. 1).

As each colony was always heterogeneous, and as this heterogeneity was preserved after many passages (we passed strain B₁ for two years), it was concluded that this variability was not a mechanical artefact (the transfer of "adhering" "light" and "dark" colonies). Evidently, the sectors in the colonies are formed

as a result of cell variability. The frequency of the appearance of "dark" cells in a "light" population was significantly greater than the opposite type of variability. Hence, greater breadth of dark sectors and their greater number were noted in comparison with light sectors in dark colonies.

We attempted to change the rate of mutation of "light" cells into "dark" ones. For quantitation, the method of counting sectors in different squares, as proposed by Kaplan (1952) and Witkin (1951), was used. It was assumed that colonies having two "halves"—a dark one and light one—had mutated at the two-cell stage, and colonies having one quarter variation had mutated at the four-cell stage, etc. Thus, by calculating the number of sectors of each kind, one could calculate the frequency of change of a single cell per generation and divide the found magnitude by the assumed number of cells corresponding to the formation in the colony of sectors of a given diameter. If this found number differed from the true frequency of mutation, then it served as a criterion for studying the effect of different compounds on the mutation rate.

For studying the effect of radioprotective compounds on the mutation rate of *B. prodigiosum*, various compounds were added to melted agar and the colonies which grew out were subsequently counted. A number



Fig. 1. The results of transferring different portions of light and dark colonies of the unstable strain of *Bacterium prodigiosum*.

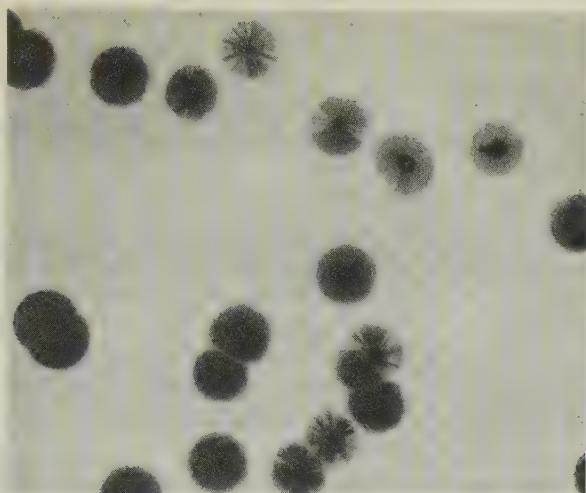


Fig. 2. Light and dark colonies of the unstable strain B₁ of B. prodigiosum.

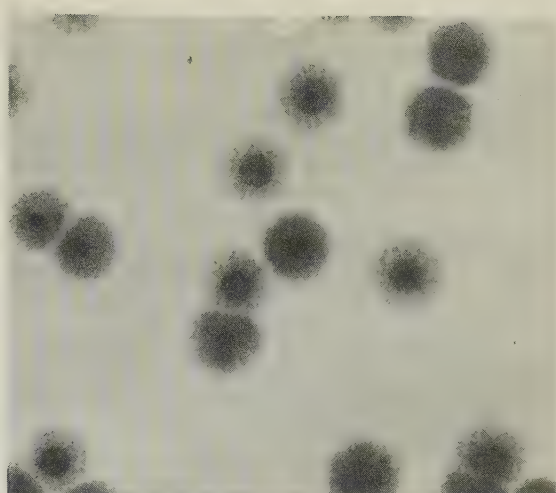


Fig. 3. Light and dark colonies of the unstable strain B₁ of B. prodigiosum on agar with sugar and 0.05% sodium hydrosulfite.

The Effect of Sodium Hydrosulfite, Thiourea and Potassium Permanganate on the Mutation Rate in Strain B₁ of B. Prodigiosum

Size of sector	Mutation rate per generation			
	control	sodium hydrosulfite (0.05%)	thiourea (0.025%)	potassium permanganate (0.0005%)
1/2	10.0±1.6 σ=3.2 n=20	0.8±0.35 σ=1.1 n=36	7.0±1.3 σ=3.4 n=27	15.2±1.8 σ=5 n=30
1/4	7.6±0.8 σ=1.6 n=20	0.7±0.23 σ=0.7 n=36	3.9±0.57 σ=1.4 n=27	9.5±1.1 σ=2.9 n=30
1/8	6.7±0.8 σ=1.7 n=20	0.7±0.13 σ=0.45 n=36	4.0±0.57 σ=1.4 n=27	8.4±0.8 σ=2.1 n=30
1/16	6.7±0.06 σ=0.1 n=20	—	—	—

of the compounds were toxic and therefore the least toxic dose was experimentally determined and this subtoxic dose was used. Sodium hydrosulfite, cysteine, unithiol (a domestic preparation which is close in structure to 2,3-dimercaptopropanol or BAL), and thiourea were tested. In addition, potassium permanganate was used as the control mutagen.

In calculating the frequency of mutation, the colonies were examined with a binocular magnifying glass. Sectors occupying 1/2, 1/4, 1/8, and in some instances 1/16 of a colony were counted. Sectors were counted in isolated colonies having a diameter of not less than 3–5 mm. The result of sector counts on 25 to 30 colonies on a single plate was used as a statistical unit.

The data from a number of experiments, at least 30, were processed by the formula for rare events. The evaluation of significant differences was based on a coefficient of probability equal to 0.95.

RESULTS

The mutation rate is given in the table under the column marked "control." The frequency of mutation

per generation for each type of sector corresponded well (aside from sectors occupying half of a colony). The large figures in the last instance can possibly be explained by that fact that in a number of colonies of "halves" two adhering cells were transferred—a light one and a dark one.

The effects of radioprotective compounds on the mutation rate are also given in the table. Sodium hydrosulfite and thiourea retarded the natural mutation rate. Sodium sulfite appeared to have an especially noteworthy effect, decreasing the frequency of mutation tenfold. The light colonies which grew on agar with sodium hydrosulfite had only very narrow dark sectors and appeared to be almost solid (Fig. 3).

No statistically significant difference was observed between the control and the organisms grown in unithiol (0.5%) or in cysteine (0.01%); therefore, these data are not given in the table. Potassium permanganate hastened the rate of mutation.

The results obtained can be explained not only as a retardation or speeding up of mutation, but also as the frequency of selection of "light" and "dark" cells.

However, if, in these experiments, there was a partial inhibition of growth of a given kind ("dark" ones), then the dark colonies on the agar with sodium hydrosulfite would be significantly less than the light ones. However, the size of all the colonies was the same, and only the size of the sectors was decreased. Consequently we are dealing with a true retardation of mutation.

DISCUSSION

The data of our experiments do not infallibly point to a relation between radioprotective compounds with an antimutagenic effect. Nonetheless, the finding of compounds possessing antimutagenic effects is not without interest. There is not much data on the inhibition of natural mutation by chemical compounds at the present time: The work of Novick and Szilard (1952) dealt with delayed mutation rates in *E. coli* with the use of adenosine. The presence of an antimutagenic effect (in relation to natural mutation) in several radioprotective compounds must be of practical as well as theoretical interest. Up to the present time the efforts of geneticists have been directed toward means by which natural variability can be increased with the aim of obtaining useful new characteristics. However, in several instances it would be useful to retard this variability (for instance, the dissociation of microorganisms in industrial situations).

SUMMARY

1. A study was carried out of the mutation rate of the unstable strain of *Bacterium prodigiosum* with no artificial treatment and under the influence of chemical compounds.

2. Certain radiation-protective compounds such as sodium hydrosulfite or thiourea reduce the rate of occurrence of natural mutations. Unithiol and cysteine do not exert such an effect. One of the mutagens (potassium permanganate) increased the mutation rate.

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ON THE ACTIVITY OF HYDROLYTIC ENZYMES IN THE ASPERGILLUS NIDULANS VARIANT PRODUCED BY IRRADIATION WITH ULTRAVIOLET RAYS

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The hydrolytic enzymes of the molds related to the *Aspergillus* and *Penicillium* genera are widely used in various industries such as the liquor industry, the tanning industry, and the antibiotics industry (Imshenetskii, 1955; Feniksova, 1958). Therefore, the problem of experimentally increasing the activity of valuable enzymes of these molds (amylase, protease, pectinase, cytase, etc.) is a very real one. Together with this, comparative studies on the activity of the enzymes in the original form of the mold and in variants obtained as a result of irradiation can open new areas on the mechanism of irradiation effects on fungal cells.

At the present time, many facts are being accumulated which show that, by means of irradiation with ultraviolet light, significantly different forms of molds can be obtained which show increased activity of a number of enzymes.

In particular, it is known that with specific doses of irradiation the formation of hydrolytic enzymes can be increased. The increased activity of the proteolytic enzymes in variants obtained from irradiation has been noted by Maxwell (1952 a,b) and Brotskaya (1956, 1958). In the opinion of Schreier et al. (1954), the increased amount of amino nitrogen in the irradiated cells can be explained by a number of factors, among which is the significant activation of proteolytic enzymes.

According to the data of Pomoshchnikova (1956), the irradiated molds contain active phosphatase. Increased amylolytic activity in the variant of *Aspergillus oryzae* obtained after irradiation was noted by Dvadsatova (1958 a,b).

However, as a rule, the activity of the oxidative enzymes and, in particular, of the enzymes which are concerned with synthetic processes in the irradiated cell, are significantly decreased, as a result of which there is retarded growth of the culture (Meisel' et al., 1955; Schreier et al., 1954).

The changes which have been noted in the behavior of different enzyme systems under the influence of irradiation depends to a significant degree on the conditions of irradiation and on the physiologic condition of the irradiated organism, as well as on the content of the medium.

The changes which occur in microorganisms under the influence of irradiation can have an inheritable character.

The present work was carried out with the aim of establishing general principles in the behavior of several hydrolytic enzymes in the mold *Aspergillus nidulans* and in a variant obtained from it as a result of irradiation, called variant n/7.

EXPERIMENTAL METHODS

The variant n/7 was obtained earlier at the Institute of Microbiology, AN SSSR, from S. Z. Brotskaya. In her published works (1956, 1958), a brief characterization of the morphological properties of this variant was given. It differs from the original form in a number of ways: retarded growth, plicated structure of colonies, decreased formation of conidiospores, etc.

Few articles have been devoted to the study of the enzymatic complex in *Aspergillus nidulans* and its variants. As a consequence of this it was necessary to make a more detailed study of the enzymatic complex in the two indicated forms of the mold.

It is known that the activity of the hydrolytic enzyme in one and the same culture of the mold can be quite variable, depending on the contents of the nutritive medium. Therefore, the activity of the indicated group of enzymes was studied on a single medium, namely, in a broth of wheat bran. This natural nutritive medium is rich and is widely used for culturing *Aspergillus* mold. For the formation of pectinase by the mold, besides the aforementioned, a carrot medium was used which is most favorable for the accumulation of this enzyme.

The wheat broth was prepared in the following manner. The bran was mixed with tap water in the ratio of 1:6 and was sterilized under a pressure of one atm for forty minutes. Then the bran was carefully mashed through three or four layers of gauze. The filtrate was then poured out in 40-ml quantities into sterile Erlenmeyer flasks of 150-ml capacity. The medium was sterilized at 0.5 atmospheres for 30 minutes. The conidia of seven-day-old cultures grown on wort agar were then transferred.

To obtain more even suspensions of conidia, the following was performed: The conidia in the thick medium were washed with a small volume of sterilized tap water, transferred into a sterile tube, and carefully shaken, after which they were filtered. The filtrate containing the conidia served as the transplanting material. The count was carried out in a

Goryaev chamber. Conidia were introduced into each tube to give 400,000 conidia per 1 ml of medium. The culture was grown at 26–28° for eight days. On the surface of the medium a dense pellicle grew which was easily separated from the culture fluid. Daily, beginning from the fourth or fifth day of the growth of the culture, a determination of the enzyme activity was carried out on the pellicles of the mold. Half of the pellicle was used for determining the activity of the enzyme, and the other half was used for determination of the dry weight. The activity of the enzyme was calculated on the basis of one gram of dry weight of the mold.

The enzyme extract for the determination of hydrolytic enzyme activity was prepared in the following manner: The pellicle of mold taken from the surface of the fluid was ground in a mortar. Then the powder was mixed with culture fluid and was filtered. The activity of the following enzymes was determined in the filtrate: protease, amylase, pectinase, and cytase.

The proteolytic activity was determined by the method of Löhlein-Volhard (1938) as modified by Babakina (1949) and by the method described by Belozerskii and Proskuryakov (1951). The first method included the following: Ten ml of the obtained filtrate was taken, and 20 ml of a 5% alkaline solution of casein which had been heated to 38° was added. The test solution was kept for an hour at 38°. After this, 10 ml of 0.2 N hydrochloric acid and 10 ml of 15% sodium sulfate solution were added and then the precipitate of the casein was filtered. In parallel tubes (control) the casein rapidly precipitated upon the addition of this amount of hydrochloric and 10 ml of 15% sodium sulfate. From the filtrate which was obtained, 25 ml of the free acid was taken up and titrated with a 0.1 N solution of sodium hydroxide with the addition of three drops of 5% purple cresol. The degree of proteolysis of the casein could be determined by the differences in the amount of alkali added during the titration to the control and experimental filtrates.*

The second method included the following: A 2% solution of casein was taken as the substrate for the determination of proteolytic activity. The casein was prepared by dissolving a known amount of it in half its volume of 0.5% Na_2CO_3 solution heated to 70°, after which a solution of 0.01 N hydrochloric acid was added until the pH was below 7. To 5 ml of the solution of casein which had been heated to 40°, 5 ml of water and 2.5 ml of enzyme extract prepared in the method described above were added. The fluid was mixed and hydrolysis was allowed to proceed at 40° for two hours. Then, from the aforementioned volume of fluid, 5 ml were taken, to which 5 ml of 4% trichloroacetic acid were added. After fifteen minutes the solution was thoroughly mixed and filtered. The transparent filtrate served for further determination of tyrosine freed during proteolysis. The calculation of results was based on tyrosine. To 5 ml of the filtrate in a 50-ml graduated test tube were added 10 ml of 0.5 N NaOH solution and 3 ml of "reactive phenol" (Belozerskii and Proskuryakov, 1951) previously diluted with two volumes of water. Then the tube was filled with water

to the 50-ml mark and was shaken; after 10 minutes of standing, a blue color developed which could be measured in a colorimeter and compared with a standard solution of tyrosine prepared in an analogous way. The smallest concentration of tyrosine which could be determined without significant error was about 0.003 mg per 1 ml. At the same time a control tube was handled analogously with the exception that trichloroacetic acid was added to the mixture of the substrate and buffer before the addition of the enzyme solution. The solution of tyrosine was prepared by dissolving a corresponding amount (0.05 g) in 0.01 N HCl.

The determination was carried out with the help of an FÉKN-54 photoelectric colorimeter.

Determination of Amylolytic Activity

This was done by titrating the amount of reduced material formed as a result of hydrolysis of starch by the enzymes. For the hydrolysis, 2 ml of a 1% starch solution and 2 ml of the enzyme filtrate were taken. The solution was mixed with the addition of 3 drops of toluene and kept for three hours at 30°. For a control, 2 ml of a 1% solution of starch was mixed with enzyme fluid and 2 ml of distilled water. After three hours of hydrolysis the amount of maltose formed was measured by the method of Bertran. To determine the sugar, 2 ml of the fluid was taken. A unit of maltase activity was determined by the amount of reduced material formed as a result of the activity of 1 ml of enzyme fluid on 1 ml of 1% starch solution in three hours.

Determination of Pectinase by the Method of Melitts (Tserevitinov, 1949)

To determine the hydrolysis of pectin, 4 ml of enzyme solution was taken and put in a tube containing 6 ml of pectin solution. A water blank served as a control. The fluid in the tubes was mixed. Three drops of toluene were added to protect against contamination. The tubes were corked and kept for 24 hours at 30°. After hydrolysis the amount of pectin remaining was determined. To 10 ml of the solution was added 100 ml of 0.1 N solution of alkali. The solution was allowed to stand overnight. Then 50 ml of 1 N acetic acid and, after 5 minutes, 50 ml of 2 N calcium chloride solution were added. After an hour of standing the solution was boiled for five minutes. The fluffy precipitate of calcium pectate was filtered through a dry suspension filter. The precipitate on the filter was washed with hot water until the silver nitrate reaction with the chloride disappeared. The filter with the precipitate was dried.

Cytase Activity

The activity of cytase was determined by the degree of hydrolysis of hemicellulose. As is known, the products of hydrolysis of hemicellulose are various monosaccharides (mannose, galactose, arabinose, and xylose). The hexoses were determined by the method of Bertran. Determination of pentose was not carried out.

*For determination of proteolytic activity it is recommended that 20 ml of the filtrate be taken and, correspondingly, that the indicated reagents be doubled in amount.

Table 1. Proteolytic and Amylolytic Activity of the Original Culture of *A. nidulans* and Its Variant n/7

Expt. no.	Culture	Culture age, days	Medium pH	Pellicle dry wt, g	Proteolytic activity				Amylolytic activity	
					amount of 0.1N NaOH, ml		amount of tyrosine, mg.		absolute amt. maltose, mg per g dry wt	amt. maltose mg per g dry weight
					absolute amt.	dry wt.	absolute amt.	dry wt.		
1	Parent	6	6.78	0.4098	1.04	2.54	0.021	0.0542	4.05	9.88
		7	7.00	0.4470	1.04	2.33	0.018	0.0403	3.44	7.70
		8	7.21	0.5082	0.98	1.92	0.018	0.0354	3.19	6.27
1	Variant n/7	6	6.50	0.3356	2.10	6.58	0.0246	0.0733	4.64	13.82
		7	6.78	0.4120	2.0	4.25	0.0198	0.0481	3.01	7.30
		8	7.25	0.4906	2.09	4.26	0.0198	0.0435	3.01	6.13
2	Parent	5	6.7	0.3079	1.15	3.73	—	—	5.69	18.48
		6	7.06	0.3655	1.15	3.15	—	—	6.16	16.85
		7	7.17	0.4250	0.94	2.21	—	—	7.08	16.65
2	Variant n/7	8	7.24	0.4542	0.73	2.07	—	—	7.50	16.51
		5	6.35	0.2085	1.46	7.0	—	—	3.85	13.36
		6	6.72	0.3039	2.29	7.53	—	—	4.85	15.95
		7	6.96	0.3903	1.76	4.50	—	—	6.25	16.01
		8	7.15	0.4578	1.41	3.73	—	—	4.78	10.44

For the analysis 6 ml of hemicellulose solution was taken which had been prepared in phosphate buffer at pH 5 to which was added 4 ml of the enzyme solution. After fixing the solution 3 drops of toluol were added as an antiseptic. The tubes were covered with corks and were left for 24 hours in a 40° incubator.

EXPERIMENTAL RESULTS

Activity of Protease and Amylase

It was earlier shown by Brotskaya (1956) that the n/7 variant of *Aspergillus nidulans* possessed significantly higher activity than the parent form. The results of the present experiments corroborate these data. In Table 1 and Fig. 1 it is seen that the proteolytic activity of the irradiated form was greater by 2 to 2.5 times than that of the parent form. In comparative studies of the amylolytic activity in the two studied forms it was shown that there was no real difference between them.

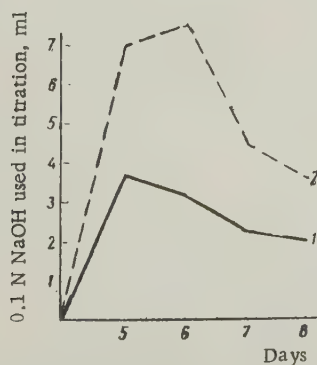


Fig. 1. Protease activity in the parent culture of *A. nidulans* and its variant n/7: 1) Parent form; 2) n/7 variant.

From data in the literature it is known that in the majority of instances no correlation is observed between the activity of the previously mentioned enzymes. On the contrary, an inverse correlation is frequently noticed (Feniksova, 1956).

As we see in variant n/7 obtained by ultraviolet irradiation there is a significant increase in the activity of the proteolytic enzymes and very little change in the activity of amylase.

Pectinase Activity

Many strains of *Aspergillus* (*A. niger*, *A. oryzae*, *A. flavus*, etc.) form active enzymes which split pectin.

The pectolytic enzymes are related to a group of adaptive enzymes. Therefore the active preparations of these enzymes can be obtained by growing molds on media containing pectin (Popova, 1935; Novotel'nov and Barkovskaya, 1935; Barinova, 1946, etc.).

Of the natural substrates, the most frequently used are carrot, apple, and sugar beet pressings, and several others.

The majority of investigators consider that the pectolytic enzymes represent a complex of the two following enzymes: 1) pectinesterase, which catalyses the hydrolytic splitting of methyl alcohol from the molecule of pectin; and 2) polygalacturonase, which catalyses the hydrolysis of polygalacturonic acid to monogalacturonic acid.

The activity of pectinase in the molds grown on wheat bran broth and also on carrot media were studied by us.

From the data given in Table 2 and Fig. 2a it is seen that the activity of pectinase after the growth of the mold on the wheat bran broth was significantly higher in variant n/7 than in the parent form of *A. nidulans*. This is expressed in the following way in the figures. The greatest amount of split pectin was

Table 2. Activity of Pectinase in the Parent Culture of *A. nidulans* and Its Variant n/7 Grown on Wheat Bran Broth

Expt. no.	Type of culture	Length of growth, days	Medium pH	Dry wt. of mold pellicle, grams	Amount of calcium pectate, mg		Amount of hydrolyzed pectin as mg of calcium pectate per g absolute dry wt.
					control	exptl. (amt. pectin hydrolyzed)	
1	<i>A. nidulans</i> (parent)	5	—	0.3489	20.4	1.9	5.45
		6	6.4	0.4450	20.4	3.6	8.09
		7	6.51	0.4953	20.4	4.0	8.08
		8	6.54	0.5859	18.4	7.9	10.06
1	Variant n/7	5	—	0.3013	20.4	11.6	38.49
		6	6.31	—	20.8	—	—
		7	6.45	0.4568	20.4	10.3	22.54
		8	6.84	0.5235	18.4	9.5	18.14
2	<i>A. nidulans</i> (parent)	4	5.51	0.2877	17.0	2.1	6.3
		5	6.07	0.3423	17.2	0.9	2.6
		6	5.79	0.4593	17.8	7.0	15.2
		7	6.36	—	17.8	4.9	—
2	Variant n/7	4	5.58	0.2500	17.0	8.4	33.6
		5	6.26	0.3455	17.2	—	—
		6	5.91	0.3707	17.8	9.7	26.2
		7	6.61	0.4408	17.8	8.4	19.0

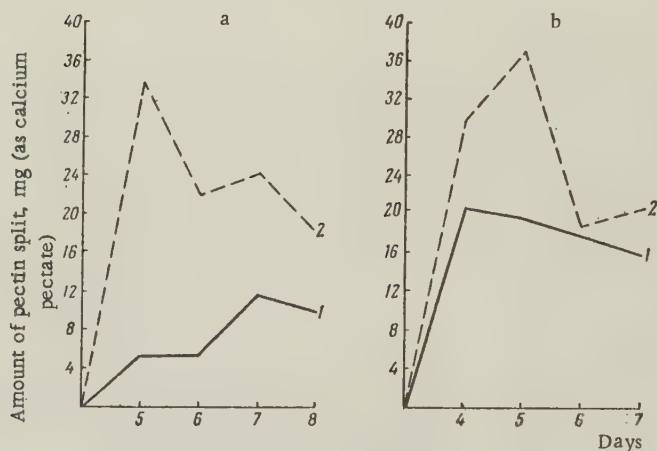


Fig. 2. Activity of pectinase in *A. nidulans* and its variant grown on wheat bran broth (a) and carrot medium (b). 1) Parent form; 2) variant n/7.

Table 3. Activity of Pectinase in the Parent Culture of *A. nidulans* and Its variant n/7 Grown on Carrot Broth

Expt. no.	Culture	Age of culture (days)	Medium pH	Pellicle dry wt, g	Amount of calcium pectate, mg		Amt. of hydrolyzed pectin as mg of calcium pectate per g absolute dry weight
					control	exptl. (amt. hydrolyzed pectin)	
1	<i>A. nidulans</i> (parent)	4	3.31	0.3744	19.3	7.0	18.7
		5	3.66	0.4168	18.9	7.5	17.99
		6	3.19	0.5286	18.3	13.8	26.1
		7	5.52	0.5657	19.9	8.9	15.73
1	Variant n/7	4	3.83	0.2811	19.3	8.4	29.88
		5	3.66	0.3624	18.9	13.0	35.87
		6	3.05	0.4564	18.3	8.5	18.62
		7	3.47	0.4459	19.9	9.6	21.56
2	<i>A. nidulans</i> (parent)	4	3.57	0.3003	19.2	7.0	23.31
		5	3.47	0.3931	19.1	7.45	18.95
		6	—	0.5184	18.25	13.70	26.42
		7	4.86	0.5712	19.80	9.0	15.75
2	Variant n/7	4	3.94	0.2660	19.2	8.4	31.57
		5	3.64	0.3570	19.1	13.0	36.41
		6	3.38	0.4618	18.25	8.5	18.40
		7	3.61	0.4823	19.80	9.65	20.00

Table 4. Activity of Cytase in the Parent Culture of *A. nidulans* and Its Variant n/7 (temperature of the experiment, 40°)

Expt. no.	Culture	Age of culture, days	Medium pH	Dry wt. of pelli-cle, mg	Amt. of hemi-cellulose split, mg	Amt. of hemi-cellulose per g dry wt.
1	<i>A. nidulans</i> (parent)	5	6.43	0.3586	46.23	128.9
		6	5.64	0.4234	81.30	192.0
		7	5.94	0.5072	82.20	162.0
		8	5.96	0.6365	76.6	120.3
1	Variant n/7	5	6.35	0.2693	36.08	133.9
		6	5.86	0.3740	82.20	219.7
		7	6.28	0.4366	75.40	172.6
		8	6.31	0.4953	81.36	164.2
2	<i>A. nidulans</i> (parent)	5	6.49	0.3410	72.83	213.57
		6	5.97	0.3983	77.58	194.76
		7	5.72	0.4332	79.92	184.41
		8	5.94	0.4810	83.90	174.51
2	Variant n/7	5	6.46	0.2759	63.10	228.60
		6	5.91	0.3295	69.30	210.15
		7	5.44	0.3963	70.77	178.56
		8	5.88	0.4267	80.27	188.10

Note: The original amount of hemicellulose was 146.0 mg.

found to result from the activity of the enzyme solution obtained from variant n/7 on the fourth day of the growth of the mold (33.6 mg of calcium pectate); the enzyme solution of the parent form gave 15.2 mg of calcium pectate on the 6th day of growth.

It was of further interest to clarify in what way activity would be increased in the forms of the mold under study if they were grown on media enriched with pectin. For this, five passages were carried out on thick carrot medium, after which the molds were grown on fluid carrot medium. The data obtained are shown in Table 3 and Fig. 2b, from which it is seen that the activity of pectinase during the growth of the parent form of the mold on the given medium was significantly higher than on the wheat broth. However, the hydrolysis of pectin by the stated enzyme occurred significantly more slowly in comparison with the activity of the analogous enzyme obtained from variant n/7. The activity of the latter was highest after five days' growth, and there was a less evident difference when the mold was grown on wheat bran broth, as opposed to carrot medium.

From these data it is evident that variant n/7 possesses greater pectinase activity than the parent form of *A. nidulans* and this enzyme, in variant n/7, is related by its nature to the group of constitutive enzymes.

Cytase Activity

The hydrolysis of hemicellulose by the fungal cytase was carried out at 40°.

From the data in Table 4 it is seen that the activity of the enzyme cytase in variant n/7 was somewhat higher than in the parent form of *A. nidulans*. Thus the amount of reduced material formed as a result of hydrolysis of hemicellulose by the enzyme solution obtained from *A. nidulans* was 193.38 mg, while the hydrolysis brought about by the enzyme solution obtained from variant n/7 gave 215.5 mg. The activity of the two investigated cultures was determined at the 6th day of their growth.

SUMMARY

1. A comparative study was carried out of the activity of hydrolytic enzymes (protease, amylase, pectinase, and cytase) in the parent strain of *A. nidulans* and in the variant n/7 obtained from it by ultraviolet irradiation.

2. It was shown that the activity of hydrolytic enzymes in the n/7 variant exhibits a general tendency towards an increase. This is particularly apparent with regard to proteolytic enzymes.

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CULTIVATION OF FLEXNER DYSENTERY BACTERIA BY THE SUBMERGED METHOD ON SEMISYNTHETIC NUTRIENT MEDIA

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The first step towards the achievement of continuous cultivation of microorganisms for the production of bacterial preparations is the use of the submerged method of cultivation. In order to change from submerged to continuous cultivation, a detailed study of the metabolism of microorganisms is necessary, to select a substrate which will be sufficiently nutrient and as far as possible free from ballast substances.

The purpose of the present investigation was to find a semisynthetic medium of the simplest composition which could be used for submerged cultivation of Flexner dysentery bacteria, type "C," for the production of enteric vaccines. Several variants of synthetic media were also tested. At the same time, the changes which took place in the amino acid composition of the media during the cultivation of bacteria were studied chromatographically.

The microorganisms were cultivated for 12 hr in conditions of intensive aeration; glucose was introduced four times into the culture, which was at the same time subjected to alkalization with 1% solution of caustic soda.

Preliminary tests, in which different variants of the media were compared, were carried out in glass vessels with a capacity of 300 ml. The selected media were then tested in large volumes in conditions which gradually approached the industrial.

The basis of all the media was the synthetic medium proposed by Troitskii, Gekker and Kovaleva, from which we eliminated amino acids. When this medium was used for the cultivation of Flexner dysentery bacteria type "C" in conditions of intensive aeration and with four administrations of glucose, it was found that the microorganisms multiplied rapidly even though the only source of nitrogen was the di-derivative ammonium citrate. However, the number of microorganisms produced (15 billion bacterial cells per 1 ml of medium) was considerably smaller than that obtained by cultivation in industrial conditions on broths. The composition of the initial medium was as follows: Na_2HPO_4 —1.0 g; KH_2PO_4 —1.0 g; MgSO_4 —1.0 g; NaCl —5.0 g; glucose—5.0 g; $(\text{NH}_4)_2\text{HC}_6\text{H}_5\text{O}_7$ —4.5 g; anhydrous Na_2CO_3 —2.0 g; nicotinic acid—0.005 g; distilled water—1 liter.

Semisynthetic media were prepared by adding to a given medium small amounts of such sources of amino acids and vitamins as hydrolyzates of casein produced by the protease action of the fungus *Aspergillus terricola*, and autolyzate of yeast.

Results of the submerged cultivation of Flexner dysentery bacteria type "C" in different variants of semisynthetic media are given in Table 1.

As will be seen in this table, in semisynthetic media containing fungal hydrolyzate of casein, the growth of

Table 1. Density of Bacterial Suspensions of Flexner Dysentery Bacteria Type "C" (No. 845) in Different Semisynthetic Media

Medium	Amt. of added substance		Number of tests	No. of cells, 10^9 /ml		
	casein hydrolyzate, %	yeast autolyzate, %		average	minimal	maximal
	15 F*		10	29.4	19	35
	5 "	"	10	23.4	17.5	27
	None	15	9	24.0	19	28
	"	5	9	24.4	18	29
	"	3	2	25.5	25	26
	"	1.5	4	24.0	23	25
	15 F*	1.5	3	33.6	31	37
	5 "	1.5	2	34.5	33	36
	5 "	1.5	6	22.0	20	26
	5 "	1.0	3	36.0	31	42
	5 "	0.5	12	28.9	25	37
	5 P*	0.5	3	29.3	29	30
Initial	5 F*	0.5	3	28.9	26	32
The same but without nicotinic acid	5 F*	0.5	12	28.3	26	32
	5 F*	0.5	3	28.5	28	29
Total			91			

*F denotes fungal hydrolyzate; P, pancreatic.

the microorganisms increased as the content of hydrolyzate was increased from 5 to 15%. The addition of different amounts (15; 5; 3; 1.5%) of yeast autolyzate to the synthetic medium had an equally stimulating effect on the growth of Flexner dysentery bacteria type "C".

On combining these ingredients, highly satisfactory results (average density of bacterial suspensions after growth of 12 hours was 28.3 billion bacterial cells per 1 ml of medium) were obtained with the initial medium containing 5% of casein hydrolyzate and 0.5% of yeast autolyzate. In this semisynthetic medium, besides the industrial strain, eight other newly isolated strains of dysentery bacteria type "C" were cultivated. The results are given in Table 2.

A chromatographic analysis of a synthetic medium containing 5% of fungal or pancreatic casein digest and 0.5% yeast autolyzate revealed 15-17 amino acids introduced into the medium with the hydrolysis.

Figure 1 is a photograph of the chromatogram made from the culture fluids of the Flexner dysentery bacteria type "C" cultivated on two series of the given medium.

As will be seen in the chromatogram, by the sixth hour of cultivation, glutamic acid and threonine (Fig. 1, spots 7 and 8), and asparaginic acid, serine, and glycine (Fig. 1, spots 4, 5 and 6) completely disappeared from the media, whereas alanine (Fig. 1, spot 9) was partially used up.

Concurrently with the semisynthetic media, certain variants of synthetic media were also tested.

As a control synthetic medium containing amino acids, we used the cystine- and tryptophan-containing medium proposed by Troitskii, Gekker and Kovaleva.

We also tested a medium containing dicarboxylic amino acids, e.g., aspartic and glutamic acids, which, judging by the changes which occur in the amino acid composition of semisynthetic media, are intensively used by the dysentery bacteria during cultivation. This is also supported by the literature.

Variants of media containing one of the dicarboxylic acids and a sulfur-containing amino acid, e.g., cystine, were also investigated.

The density of the bacterial suspensions of Flexner dysentery bacteria type "C" (No. 845) in different synthetic media is shown in Table 3.

As will be seen from Table 3, the addition of a mixture of tryptophan and glutamic acids to a synthetic medium had a slight stimulating effect on bacterial growth. A somewhat better result was produced by adding a mixture of cystine and tryptophan. The best results, however, were obtained when a mixture of cystine and one of the dicarboxylic amino acids was added.

A chromatographic analysis of the synthetic media during the cultivation of Flexner dysentery bacteria type "C" showed pronounced changes.

By the sixth hour of growth, aspartic acid had completely disappeared and glutamic acid had partially disappeared from media containing dicarboxylic acids.

Table 2. Density of Bacterial Suspensions of Newly Isolated Strains of Flexner Dysentery Bacteria Type "C" in Semisynthetic Media

Strain no.	No. of tests	Number of cells, 10^9 /ml		
		average	minimal	maximal
845	12	28.3	26	32
55	2	28.5	28	29
315	2	29.5	29	30
3913	2	24.5	24	25
331	4	23.0	21	25
6	2	24.5	24	25
2691	2	24.0	24	24
590	2	22.5	21	24
829	4	21.0	20	22
416	2	20.0	17	23

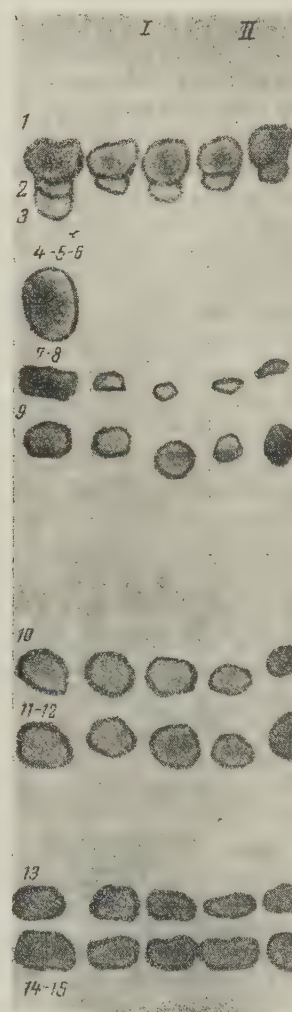


Fig. 1. Changes occurring in the amino acid composition of a semisynthetic medium containing 5% fungal casein hydrolyzate and 0.5% yeast autolyzate, during the cultivation of Flexner dysentery bacteria type "C" in conditions of aeration. Solvent: butanol + acetic acid + water. I—First test; II—second test; left column—control. 1) Cystine; 2) lysine; 3) arginine; 4) aspartic acid; 5) serine; 6) glycine; 7) glutamic acid; 8) threonine; 9) alanine; 10) tyrosine; 11) methionine; 12) valine; 13) phenylalanine; 14) and 15) leucines.

Table 3. Density of Bacterial Suspensions of Flexner Dysentery Bacteria Type "C" (No. 845) in Synthetic Media with Different Amino Acid Compositions

Medium No.	Amino acid composition of media	No. of tests	Number of cells, 10^9 /ml		
			average	minimal	maximal
3	None	19	15.5	4	23
11	Aspartic and glutamic acids	12	17.5	12	24
1	Cystine, tryptophan	12	19.45	12	23
12	Cystine, aspartic acid	9	23.1	21	25
13	Cystine, glutamic acid	11	23.3	21	26

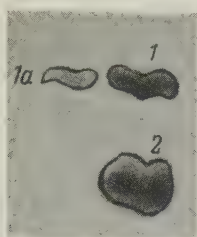


Fig. 2. Chromatogram of the synthetic medium without amino acids at different hours of cultivation of Flexner dysentery bacteria type "C" in conditions of aeration. Solvent: butanol + acetic acid + water. 1, 1a) Glutamic acid; 2) alanine.

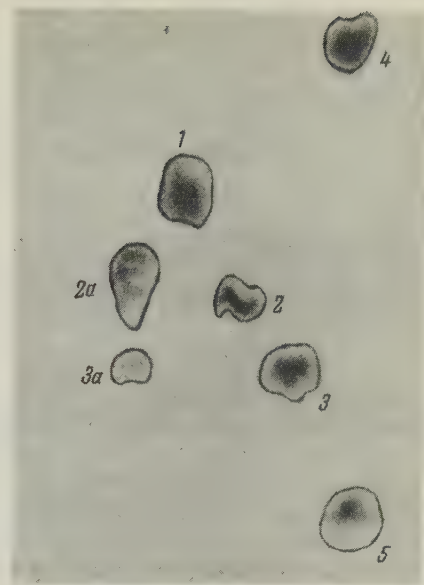


Fig. 3. Chromatogram of the synthetic medium No. 1 (Troitskii, Gekker, Kovaleva) at different hours of cultivation of Flexner dysentery bacteria type "C" in conditions of aeration. Solvent: butanol + acetic acid + water. Control on the left. 1) Aspartic acid; 2, 2a) glutamic acid; 3, 3a) alanine; 4) lysine; 5) α -aminobutyric acid.

By the twelfth hour of cultivation, the glutamic acid spot from six-hour specimens, which had turned pale on the chromatograms, again became bright in color. Glutamic acid was also found in media which did not contain it at the beginning of the test. Alanine also was found in practically all the media.

Apparently, during the cultivation of bacteria in synthetic media which contained either no amino acids or only small amounts, synthesis of amino acids took place along with the intensive decomposition of the nutrient substances. In the given media, the sources of glutamic acid formation were probably glucose and ammonium citrate. Alanine formation in the medium was probably the consequence of the processes of transamination between dicarboxylic amino acids and pyroracemic acid as well as a result of the processes of amination of pyroracemic acid. In a medium containing tryptophan, alanine may also occur as a product of the disintegration of tryptophan.

The chromatograms (Figs. 2 and 3) show glutamic acid spots in a medium without amino acids (Fig. 2, spot 1a; Fig. 3, spot 2a) and alanine spots (Fig. 3, spot 3a) in a medium containing cystine and tryptophan.

SUMMARY

1. A satisfactory growth of Flexner type "C" dysentery bacteria was obtained by the submerged method on a semisynthetic medium containing 5% fungal or pancreatic casein digest and 0.5% yeast autolyzate.

2. Good results were likewise obtained on a synthetic medium containing one of the dicarboxylic amino acids and cystine.

3. In the tested synthetic media (both with and without small amounts of amino acids) a synthesis of glutamic acid and alanine was chromatographically demonstrated in submerged cultures of Flexner type "C" dysentery bacteria.

THE ORIGIN AND CHEMICAL COMPOSITION OF THE EXTRACELLULAR SECRETIONS OF AZOTOBACTER CHROOCOCCUM

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The secretion of nitrogenous substances into the surrounding medium by normally growing azotobacter cells has been noted by many investigators (Kostychev et al., 1926; Vinogradskii, 1952; Novogradskii, 1933; Roberg, 1936; Butkevich and Kolesnikova, 1934; Fedorov, 1952a; Rabotnova and Glagoleva, 1953; Rabotnova and Rodionova, 1953; Kurdina, 1955, etc.). However, neither the quantitative nor qualitative aspects of this phenomenon have been adequately studied to date, despite the fact that these questions are of considerable interest both from the point of view of the chemistry of the fixation of molecular nitrogen by these microorganisms and its practical utilization in agriculture as a bacterial preparation (azotobacterin). This circumstance prompted us to undertake a more detailed study of these questions.

In the first series of experiments, we studied the quantitative aspect of the secretion of nitrogenous substances when *Azotobacter chroococcum* (strain 53) was grown under the conditions of the usual laboratory culture.

For this purpose, the microorganism under investigation was cultured on liquid nitrogen-free nutrient medium as modified by Fedorov. Glucose in a concentration of about 0.1 mole was used as the carbon source. Substances stimulating the secretion of nitrogenous compounds into the medium were not used in this experiment, but cultivation of the azotobacter was terminated at various stages of development. At the end of the experiment, the cells which had grown were separated from the culture fluid by centrifugation at 3500 rpm. The centrifugate obtained was then filtered through a Chamberland bacterial filter for the complete removal of cells, the absence of which from the filtrate was established by means of plating the filtrate on the same nitrogen-free agar medium. The absence of filterable forms of azotobacter was checked by plating the filtrate on Fedorov's medium with 0.1% KNO_3 or asparagine, since, according to Novogradskii's data (1935), these forms do not germinate on nitrogen-free media. We regarded this check as necessary, because there have been indications in the literature concerning the presence of filterable forms in azotobacter (Regel, 1932; Rybalkina, 1938), the presence of which in the filtrate was undesirable, inasmuch as the nitrogen which they contain might have been mistaken for the nitrogen of azotobacter secretions and

could have given increased yields of extracellular nitrogen. Neither viable azotobacter cells nor filterable forms were found in our filtrates.

In the experiment given below, the cultivation of *A. chroococcum* (strain 53) was terminated after 3, 6, and 13 days. In the last interval of the experiment, the glucose in the medium was almost completely utilized. When the experiment was finished, the unused glucose in the medium was determined (according to Bertrand), as well as the total nitrogen of the azotobacter cells and of the medium (according to Kjeldahl).

The results of these analyses are given in Table 1.

From these data, it is easily established that nitrogenous substances appeared in the azotobacter cultures even before the exhaustion of the energy-yielding substance of the medium. Even in three-day cultures, which had utilized only 25% of the energy-yielding substance added, 13.7% of fixed nitrogen was found in the composition of the nutrient medium. As the energy-yielding substance was further utilized, the absolute quantities of the nitrogenous substances secreted into the medium rose sharply (from 1.14 mg to 1.82 mg), but in comparison with the total amount of nitrogen fixed, they remained nearly level and were in the range of 10%. As the culture aged, their relative amounts even dropped somewhat.

Similar results were also obtained in another experiment where the intervals of cultivation of *A. chroococcum* were longer and, in addition, the ammonia-nitrogen content of the filtrate from cultures of this microorganism was determined separately (Table 2).

These data show that the relative amount of nitrogenous substances secreted into the medium by azotobacter did not exceed 10-15% of the total amount of fixed nitrogen in this experiment as well. After the exhaustion of the energy-yielding substance in the medium, it not only did not increase, but even tended to decrease somewhat. On the basis of the data given, it must be considered that the nitrogenous substances secreted are not products of the autolysis of dead azotobacter cells. Otherwise, their amount should have risen sharply after the utilization of the energy-yielding substance. Therefore, there is every reason to assume that the nitrogenous substances found in the medium are produced during the course of growth of azotobacter and are a sort of "waste product" of the

Table 1. Total Nitrogen Content of the Nutrient Medium and Cells of *A. chroococcum* as a Function of Age of the Culture

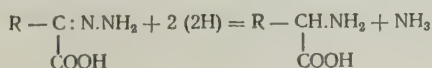
Culture age, days	No. of cells, 10 ⁹ /ml	Glucose utilized, g	Intensity of glucose utilization, %	Total fixed N in culture, mg	N in azotobacter cells, mg	Total N of filtrate, mg	Filtrate N, % of total culture N
3	33.6	0.47	22.3	5.75	4.96	0.79	13.7
6	124.8	1.09	53.2	10.39	9.25	1.14	11.0
13	155.4	2.05	100.0	20.97	19.15	1.81	8.7

Table 2. The Secretion of Nitrogenous Substances into the Medium by *A. chroococcum* as a Function of Age of the Culture

Culture age, days	Glucose consumed during experiment, g	Intensity of glucose utilization %	No. of cells that grew, 10 ⁹ /ml	Total fixed N, mg	N content of cells, mg	Total N in medium filtrate, mg	NH ₃ -N in filtrate, mg	Filtrate N, % of total N
5	0.51	25.5	117.9	8.27	7.0	1.27	0.22	15.4
10	1.56	78.0	183.2	19.23	17.22	2.01	0.55	10.5
15	1.91	95.8	367.8	24.95	21.47	3.48	0.67	14.0
20	2.00	100.0	214.3	21.01	18.46	2.55	0.94	12.1
25	2.00	100.0	174.9	26.53	23.8	2.73	1.03	10.3

synthetic activity of the given microorganism. In this respect, growing azotobacter cells behave similarly to plant cells which, as is well known, also secrete organic and mineral substances into the surrounding medium through roots and leaves. It is these secretions which serve as food for various epiphytic bacteria growing on the surfaces of plants. The difference between azotobacter and plants is only that in cultures of azotobacter, the relative amount of extracellular secretions per unit of cell substance synthesized is somewhat greater than in plants. Furthermore, it is curious to note that azotobacter secretes chiefly nitrogenous substances into the medium, although it synthesizes them with the utilization of such a poorly available source of nitrogen as molecular nitrogen.

The chemical nature of the nitrogenous secretions of azotobacter varies, but about 15–20% of the extracellular nitrogenous secretions (Table 2) consists of ammonia nitrogen. Inasmuch as this compound appears in the very first stages of development of the given microorganism, it must be assumed that it is formed as a side-product during the course of nitrogen fixation rather than being formed as a product of autolysis. Ammonia is secreted in definite proportionality with respect to the amount of molecular nitrogen fixed, which serves as additional proof that ammonia is formed as the result of side reactions which are not connected with the direct reduction of molecular nitrogen by active hydrogen (Kostychev et al., 1926; Vinogradskii, 1952; Wilson and Burris, 1947). The secondary but regular formation of ammonia inevitably follows from Fedorov's scheme of the process of nitrogen fixation, in which it is assumed that the hydrazine derivatives of keto acids are reduced to amino acids by active hydrogen with the parallel production of ammonia. This reaction must proceed according to the following scheme:



It is during this reaction that some amount of free ammonia is produced which, due to its rapid diffusion, is apparently given off to the outside of the cell. If its appearance is really connected with this process, then ammonia should be produced in amounts approximately equal to the units of nitrogen utilized by the microorganism for the synthesis of cellular material. This requirement was met experimentally. In the first three intervals of cultivation, when the energy-yielding material was beginning to be used up from the medium but was still not completely exhausted, and the processes of the synthesis of cellular material were going on at a normal rate, the ratio between nitrogen fixed and ammonia nitrogen was about 30:1. Only after the exhaustion of the energy-yielding substance did it shift to 20:1, which indicates the partial secretion of ammonia due to the processes of autolysis as well.

All of these data show that the extracellular secretions of azotobacter are basically "waste products" of its synthetic activity and apparently arise during the reduction of the hydrazine derivatives of keto acids to amino acids. A smaller part, however, is apparently connected either with the incomplete reduction of these derivatives to amino acids, or with the incomplete utilization of the amino acids formed for further protein synthesis. The latter possibility may be associated with the fact that when hydrazine is accepted from the nitrogen-fixing enzyme, pyruvic and oxalacetic acids, which are formed more easily than other keto acids during glucose oxidation, are utilized more extensively. In this case, large amounts of alanine and glutamic acid would be produced from them. When these substances are produced in great abundance, part of them may remain unutilized during protein synthesis and may be secreted into the surrounding medium as "waste products" of the synthetic activity of the microorganism. This is apparently why, along with ammonia, alanine accumulates in the medium (in young cultures), or alanine and glutamic acid, which are included (along with proline) in the composition of the simplest pep-

Table 3. The Chemical Composition of the Extracellular Nitrogenous Secretions of *A. chroococcum*

Age of culture, days	Total N of filtrate per 100 ml of medium, mg	Amino acid N, mg		Ammonia nitrogen, mg	Amino acids on chromatograms after hydrolysis
		Before hydrolysis	After hydrolysis with 20% HCl		
3	0.79	0.34	0.36	0.0	Alanine
6	1.14	0.0	1.10	0.21	Alanine, glutamic acid, serine, proline
13	1.81	0.0	1.55	0.28	Alanine, glutamic acid, tyrosine, and one unidentified amino acid

Note. The biuret reaction before hydrolysis was negative in all cases.

tides, accumulate (in older cultures). This sequence of processes is confirmed by the data in Table 3. In the experiment, ammonia was determined by the diffusion method in Conway plates, while amino acids were determined by the Van Slyke method (before and after hydrolysis with 20% hydrochloric acid) and by paper chromatography. Prior to chromatography, the amino acids were extracted with acetone acidified with hydrochloric acid according to Lissitzky's method (1954).

Simple peptides composed of several amino acids apparently constituted the major part of the nitrogenous substances in the extracellular secretions of azotobacter. Alanine and glutamic acid occupied a prominent position among these amino acids. These amino acids are easily determined after hydrolysis of the filtrate with 20% hydrochloric acid. Without hydrolysis, however, they could not be detected in the filtrates of 6- and 13-day cultures. Only three-day cultures contained alanine. Since this amino acid was also found in all hydrolyzates, it must be assumed that it is a constant component of the nitrogenous secretions of azotobacter.

The quantitative amino acid content of the filtrates of cultures grown for different periods of time proved to be different, and clearly increased as the age of the culture increased. Whereas in 6- and 13-day cultures the nitrogen of the amino acids released during hydrolysis constituted about 80–85% of the total nitrogen of the filtrate, in young three-day cultures it did not rise above 50%, but the main portion of this nitrogen was accounted for by free amino acids. The other half of the nitrogen in filtrates of young cultures was possibly composed of hydrazine derivatives, but their quantitative determination is extremely difficult due to the lack of suitable methods for detecting these compounds. We did not find free hydrazine, hydroxylamine, and oximes in azotobacter filtrates, however.

The nitrogenous substances present in filtrates of *A. chroococcum* cultures must be regarded as vital secretions of this organism, because they are represented by relatively simple compounds among which the most prominent are ammonia, certain amino acids, and the simplest peptides, consisting of approximately four amino acid residues. If the nitrogenous secretions of azotobacter were products of the autolysis of its cells, larger fragments of protein molecules giving a positive biuret reaction should also have been found in the culture filtrates. However, in our experiments, the biuret reaction with the filtrates of azotobacter

cultures was always negative. Aside from this, if autolytic products were present in the filtrates, the amino acid composition of the hydrolyzed filtrate would have been more varied, since it was shown by the analyses of Bukatsch, Bürger, and Schlüter (1956), as well as those of Belozerskii, Zaitseva, and Tyuleneva (1958) that the composition of the proteinaceous substances of azotobacter includes from 17 to 20 different amino acids. Finally, the origin of the nitrogenous substances in filtrates of azotobacter cultures is not associated with autolytic processes because their quantity not only does not increase in old cultures, but remains approximately the same after the energy-yielding substance is used up. All of this is evidence of the fact that autolytic processes proceed very slowly in azotobacter cultures, and that in young cultures which are fixing molecular nitrogen vigorously, they do not take place at all. Therefore, the extracellular nitrogenous secretions of this microorganism must be regarded as the "waste products" of its synthetic activity and related to the products arising in the course of secondary reactions accompanying the process of nitrogen fixation.

SUMMARY

1. Filtrates of *Azotobacter chroococcum* (strain 53) cultures grown on Fedorov's modification of liquid nutrient medium with 2% glucose consistently contained nitrogenous compounds, which constituted from 9 to 15% of the total amount of nitrogen fixed.

2. In young, three-day cultures, ammonia and free amino acids (alanine) comprised the largest amount of the nitrogenous substances of the filtrate, while in older cultures, ammonia and the simplest peptides or related compounds, consisting mainly of four amino acids among which alanine and glutamic acid were predominant, constituted the largest amount.

3. The nitrogenous substances in the filtrates of azotobacter cultures must be related to the products of the vital secretion of this microorganism and regarded as "waste products" of its synthetic activity, because they appear in the medium in amounts strictly proportional to the units of nitrogen fixed. They arise as the result of secondary reactions apparently associated with the reduction of the hydrazine derivatives of keto acids to amino acids, and can serve as additional proof of the correctness of M. Fedorov's contention that the process of nitrogen fixation is associated with the formation of hydrazines as intermediate products of the reduction of molecular nitrogen.

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SOME PECULIARITIES OF RHIZOBIUM LUPINI AND RHIZOBIUM JAPONICUM

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In our investigations, it has been observed that nearly all soils on which soybeans are cultivated do not contain Rhizobium japonicum. However, after growing soybeans for a long time on the same soil over a period of several generations, root nodules appear on the soybean roots. In the work which we carried out earlier, we obtained root nodules on soybeans in the third and fourth generations under control conditions on soil containing a natural population of all species of Rhizobium except R. japonicum as well as on soil enriched with active strains. In this case, along with root nodules containing strains of R. japonicum, root nodules formed on the soybeans from which strains differing from R. japonicum in morphological-physiological and cultural characteristics were isolated. All of the strains differing from strains of R. japonicum gradually lost their properties of virulence with respect to soybeans in subsequent generations. Only strains corresponding in virulence, activity, and physiological properties to strains of R. japonicum retained their virulence with respect to soybeans in subsequent generations.

Characteristically, all of the strains obtained which retained virulence and the other properties indicated for a long time were similar to the soil population of R. lupini.

These results led us to the idea of studying the peculiarities of R. lupini and R. japonicum for the purpose of establishing their interrelationship as well as the pathways of their phylogenetic development. From the results presented, the hypothesis can be made that in soils which do not contain strains of R. japonicum

the formation of such strains occurs, and the question is only in whether the formation of these strains of R. japonicum occurs by chance or according to definite patterns.

In order to elucidate this, greenhouse experiments were set up in sterile cultures on quartz sand and in Helriegel solution..

The soybean seeds were sterilized in 0.1% HgCl₂ solution for 3-5 minutes, after which they were washed in pure 96% alcohol, and then several times in sterile distilled water until all traces of chlorine were completely gone. The sterility of the seeds prior to inoculation was checked on Demolon's nutrient medium, as well as on MPB, for root-nodule bacteria. The experiments were conducted in 1955, 1956, 1957, and 1958. For the experiments, we took 30 strains each of R. lupini and R. japonicum of various origins: from Yugoslavia, the Soviet Union, America, and Japan. In planting the first generation, the soybean seeds were inoculated with strains of R. lupini, while lupine seeds were inoculated with strains of R. japonicum. Each experiment was set up with five repetitions. The plants were grown for several days in the same containers until the appearance of root nodules. As the plants stopped blooming, four repetitions were taken from each generation and new plantings were made immediately. In the plants selected, the root nodule content was analyzed, and the weight of the whole plant and of its separate organs was measured. The plants of the fifth repetition were analyzed in the ripening phase. There were two controls in each experiment: the first one without inoculation, and the second with inoculation by a typical strain of Rhizobium.

The Virulence of R. lupini Strains with Respect to Soybean Plants

Strains of <u>R. lupini</u>	Gen. I	Generation II		Generation III		Generation IV	
	No. of plants with nodules, %	No. of plants with nodules, %	No. of nodules per plant	No. of plants with nodules, %	No. of nodules per plant	No. of plants with nodules, %	No. of nodules per plant
1. K3; K2; B1; B2; B8; B10; B13; B17; N88; N67; B45; N55; N52; N37	0	0	0	100	20-28	—	—
2. Ja1; Ja8; Am3; Am28; Am34; Mc5; Mc12; Mk8	0	0	0	100	17-32	—	—
3. M3/IV; M1/V	0	90-100	18-25	—	—	—	—
4. L359; L36a; P24/1; P82/4; B18/11; B38/11	0	0	0	5-10	5-8	100	12-22

The results obtained after inoculation of the soybeans with strains of *R. lupini* during four generations are shown in the table, from which it is seen that, after several generations, the *R. lupini* strains acquired the ability to form root nodules on soybean roots. This took place with all of the *R. lupini* strains tested. The largest number of strains formed root nodules on soybean roots in the third generation, while the least number formed root nodules in the fourth generation, and only two strains of *R. lupini* formed root nodules at the end of the second generation. These two strains differed from the rest of the strains not only in their origins, but also represent separate ecotypes of *R. lupini*. These strains were collected from the southern soils of Yugoslavia; their main characteristic is their more rapid adaptability as compared with the rest of the *R. lupini* strains. Such ecotypes of *R. lupini* are contained in the soils from warm regions, where large numbers of plants from the *Vigna* sp. group are grown. On the basis of the results obtained, it can be concluded that strains of *R. lupini* change during the course of 3-4 generations under the influence of the new plant, adapting to the new host, and acquiring the ability to penetrate the root and to form root nodules on the roots of the plant (Figure).

Can the formation of root nodules on soybean roots under the influence of *R. japonicum* strains be regarded as the result of newly acquired virulence with respect to the new plant host, i.e., were *R. japonicum* strains formed from strains of *R. lupini*? In order to answer this question, we studied the properties of virulence with respect to soybeans of cultures isolated from already-formed root nodules over a period of two years. At first, the cultures were replanted every seven days in order to obtain the most distant generations possible. However, even after two years of testing, the soybeans retained the property of forming root nodules on their roots, i.e., all of the strains remained virulent and active with respect to the new host.

On the basis of this, we believe that, under the influence of several generations, the *R. lupini* strains changed, adapted to the new plant, and developed new virulence with respect to soybeans, i.e., acquired a property characteristic for *R. japonicum* strains.

In our opinion, this manner of formation of *R. japonicum* strains goes on continuously in all soils in which soybeans are cultivated. Under the influence of soybean plants, strains of *R. japonicum* are continually being formed from *R. lupini*. The proof that the formation of *R. japonicum* strains occurs under the influence of soybean plants is the fact that *R. japonicum* is found only in soils on which soybeans have already been cultivated. At the same time, all the cultivated soils which we investigated always contained *R. lupini*. Apparently, over a period of three generations of growth of soybeans, a large number of generations of *R. lupini* develop which, under the influence of soybeans, gradually change from generation to generation. After



The production of root nodules on soybean roots by modified strains of *Rhizobium*.

the penetration into the root of the new plant host, new strains, namely, strains of *R. japonicum*, finally arise.

Thus, we studied the mode of origin of *R. japonicum* strains which has occurred regularly and is occurring now during the cultivation of *Glycine hispida*. This circumstance points to the genetic relationship between strains of *R. lupini* and *R. japonicum*.

It is therefore entirely understandable that strains of *R. japonicum* and *R. lupini* are extremely similar in their morphological and physiological indicators and properties. Meanwhile, if the formation of a particular strain of *R. japonicum* or *R. lupini* is observed and their physiological properties and peculiarities of growth are compared, certain changes can be noticed.

These changes are largely of a physiological character, and pertain to the rate of growth. The majority of *R. japonicum* strains grow somewhat more rapidly than the initial strains of *R. lupini*, as the result of which the growth properties of the soybeans also change. In all strains of *R. japonicum*, the cells are not closely connected with one another, which is a function of the better growth of the cultures and better utilization of carbon. In addition to this, they change the reaction of the medium more slowly, especially on peptose media, as the result of which better growth of *R. japonicum* strains occurs on these media.

SUMMARY

1. It was shown that under the influence of the root system of soya, strains of the root nodule bacillus *Rhizobium lupini* are modified in such a way that after several generations they become virulent for soya.

2. The modified strains of *R. lupini* are identical in cultural and morphological-physiological properties to *Rhizobium japonicum*.

THE SELECTIVE UTILIZATION OF BUTYRIC ACID BY SOME AEROBIC SOIL MICROORGANISMS

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The indications in the literature (Rode, 1955) concerning the insignificant content of low-molecular fatty acids in cultivated soils is evidence of the fact that butyric acid, which is constantly being formed in the breakdown of organic substances in the soil, in turn rapidly becomes subject to further biological breakdown. The range of microorganisms with proven capacity to utilize butyric acid is quite wide. Under conditions of limited access of air, butyric acid can be utilized by certain agents of the processes of denitrification, sulfate reduction, methane fermentation, and sulfur oxidation. Under aerobic conditions, various fungi, yeast-like microorganisms, nitrogen-fixing bacteria, acetic acid bacteria, and a variety of spore-forming bacteria which grow well on meat-peptone media may serve as decomposers of butyric acid (Bredemann and Werner, 1932; Werner, 1933).

In the course of the methodical work which we carried out, it was established that aerobes which grow at a neutral reaction of the medium predominate among the soil microorganisms that utilize butyric acid.

The number of aerobic butyrate-decomposing microorganisms represented by actinomycetes and a few bacterial forms can be expressed in millions of cells per 1 g of soil. In the present communication, information is presented on the butyrate-decomposing activity of a number of cultures isolated from the soil and the peculiarities of their carbon nutrition.

MATERIALS AND METHODS

Agar medium made of fermented starch, which was prepared according to a method* described earlier, was inoculated with 10^{-5} dilutions of various soil samples. This medium contains about 0.3% volatile fatty acids, consisting of a mixture of butyric and acetic acids in the ratio of 2.0-3.0:1. Under the microscope, under low magnification, colonies were noted with rapid and profuse deposition of calcium carbonate crystals (Fig. 1) as an indicator of the decomposition of organic acids. Selected colonies were subcultured on agar slants of the same composition. In the process of obtaining pure cultures, it was noted that when actinomycetes were stored for a long time, a wide free zone appeared between the colony and the ring of crystals; this zone was absent in bacterial colonies (Figs. 2 and 3). The pure cultures isolated were subjected

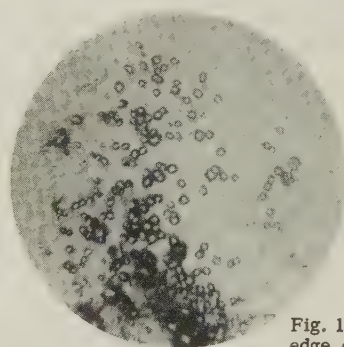


Fig. 1. Deposition of CaCO_3 crystals at edge of bacterial colony on fermented starch agar. Magnification 70 $\times$.

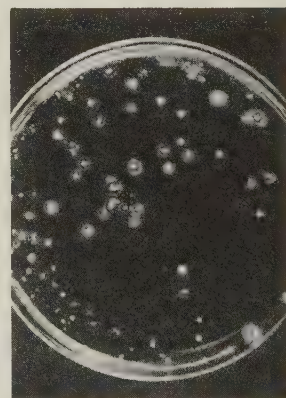


Fig. 2. Bacterial colonies surrounded by CaCO_3 crystals on fermented starch agar.



Fig. 3. Actinomycete colonies surrounded by CaCO_3 crystals on fermented starch agar.

*Bychkovskaya (196)

Table 1. Comparative Activity of Butyric and Acetic Acid Utilization by Pure Cultures (after seven days of growth, in % of initial content of acids in medium)

Group	Culture no.	Fermented starch medium		Synthetic media			
		butyrate	acetate	I		II	III
				butyrate	acetate	butyrate	acetate
I	13	0	100	—	—	No growth	—
	23	0	58	—	—	—	—
	7	62	51	17	12	—	15
II	17	—	—	16	25	No growth	29
	11	53	69	—	—	—	41
	24	23	75	—	—	—	23
III	1	50	6	53	15	91	76
	3	76	9	46	30	77	79
	21	43	82	40	100	82	74

to the first preliminary selection according to the rate and intensity of growth on the given medium. Inasmuch as growth on this medium could have been due to acetic acid alone (rather than butyric), a second selection was further made by inoculating the cultures on synthetic medium of the following composition (in %): potassium or sodium butyrate—0.2; KH_2PO_4 —0.05; CaCl_2 —0.01; MgSO_4 —0.01; NH_4NO_3 —0.1; NaCl , FeSO_4 , and MnSO_4 —traces; pH 7.0–7.2. The utilization of acids was determined by the Duclaux method after growing the cultures for 7–10 days on liquid media of the same composition.

In the experiments, media made of fermented starch were used: I) synthetic medium with a mixture of acetate and butyrate; II) synthetic medium with butyrate alone; III) synthetic medium with acetate alone.

EXPERIMENTAL

1. Comparative Activity of Butyric and Acetic Acid Utilization by Pure Cultures

By the method described above, 35 cultures were isolated, of which 18 remained after the first selection: 13 bacterial cultures and 5 cultures of actinomycetes. This ratio was the result of the purposeful selection of bacterial cultures; in actual fact, actinomycete colonies predominated over bacterial colonies in the overwhelming majority of cases in inoculations from soil.

Quantitative tests of activity showed that the selected cultures fall into three groups: I) bacteria which do not utilize butyric acid (two cultures); II) microorganisms which utilize butyric acid either on medium made of fermented starch, or on synthetic medium under conditions where acetate is added to the latter (eight cultures of bacteria and five cultures of actinomycetes); III) bacteria which actively utilize butyric acid regardless of the presence of acetate in the medium (three cultures). Data are given in Table 1 on the activity of the cultures in groups I and II and (selectively) of part of the cultures in group III.

The activity of acetic acid utilization on synthetic medium containing sodium acetate as the sole carbon source is shown in the extreme right column of Table 1.

The data obtained show that many soil microorganisms require available acetate in order to exhibit the ability to utilize butyric acid. This conclusion is of great ecological interest in connection with the fact

that acetic acid always accompanies butyric acid fermentation.

Along with this, the existence in the soil, albeit in smaller quantities, of special aerobic microorganisms with a specific relationship to butyric acid has been demonstrated. These microorganisms (group III bacteria) have a high butyrate-decomposing activity, and do not require the presence of available acetate in the medium to exhibit it. The group III bacteria are also ahead of the others with respect to vigor of consumption of the acetic acid salts present in the medium as the sole carbon source (Table 1).

From an analysis of the data presented, it is seen that the high activity of acid utilization by group III bacteria was manifested in the presence of a single volatile acid in the medium; in the presence of both acids, however, the energy of consumption of acetic acid decreased in cultures Nos. 1 and 3, while in culture No. 21, the energy of consumption of butyric acid decreased. This unusual behavior of the cultures was confirmed in further experiments, in which the dynamics of consumption of the acids was studied when they were both present in the medium. It is seen from Fig. 4 that cultures Nos. 1 and 3, in distinction from culture No. 21, utilized butyric acid first rather than acetic. The insignificant reduction in acetate concentration after 14 days of cultivation may also have been the result of the accumulation of acetic acid in the medium as an end product of butyric acid decomposition. There are statements in the literature (Kleinzeller and Bass,

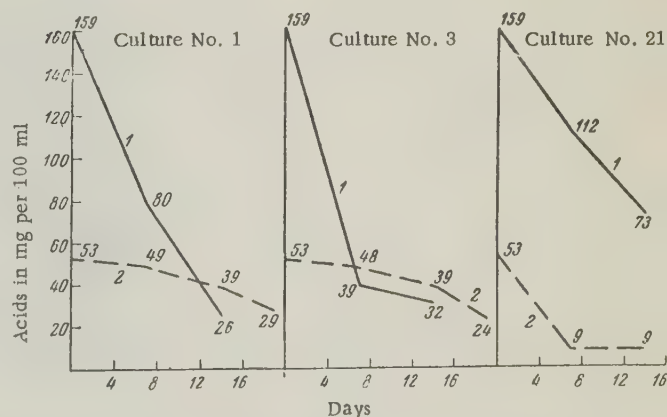


Fig. 4. Dynamics of utilization of volatile acids by active cultures in fermented starch medium. 1) Butyric acid; 2) acetic acid.

1948) concerning the possibility of a similar course of aerobic oxidation of butyric acid salts, and this has been experimentally demonstrated for certain streptococci (Wolin, Evans, and Niven, 1952). However, there is little basis for such an assumption in the cases of cultures Nos. 1 and 3, inasmuch as acetic acid was never detected in any noticeable amounts during cultivation in media containing butyrates as the sole carbon source.

2. Characteristics of Active Cultures

A brief description of the most active cultures (group III) is given.

a) Cultures Nos. 1 and 3. Nonmotile, Gram-negative, nonsporogenous, slightly curved rods (Figs. 5 and 6). Typical mycobacteria according to growth cycle. Colonies slimy, spreading; No. 1, dirty white with slight straw-colored tint; No. 3, yellowish-orange. Profuse growth on meat-peptone media with slow formation of ammonia, but without production of H_2S , indole, and other foul-smelling products. Profuse growth on potato slices. Slow preptonization in milk. Culture No. 3 liquefies gelatin. Growth (with acidification of the medium) on maltose, sucrose, glucose, ethanol, glycerol,

and mannitol in synthetic medium with NH_4NO_3 and mineral salts. Profuse growth with alkalization of medium on synthetic medium containing the salts of succinic, malic, and benzoic acids. Culture No. 1 isolated from marshy soil; culture No. 3, from well-cultivated podzol soil.

b) Culture No. 21. Nonmotile, nonsporogenous coccobacterium, coccus when mature (Fig. 7). It was not possible to get a distinct Gram stain. Colonies semitransparent, with strong fluorescence; homogeneous, with smooth edges. Does not alter milk or gelatin. Good growth on meat-peptone media with weak ammonia production. Utilizes the same forms of carbon as the preceding cultures with the exception of glucose and benzoates. Isolated from a fresh specimen of hot-house soil.

None of the three cultures grow on cellulose, pectin, starch, dextrin, lactose, galactose, or arabinose. They assimilate ammonium salts and nitrates in the presence of acetates and butyrates. The reduction of nitrates proceeds through the nitrite stage, but true denitrification is absent. Urea, amino acids, asparagine, and peptone can serve as sole sources of nitrogen and carbon. Obligate aerobes. Optimum pH

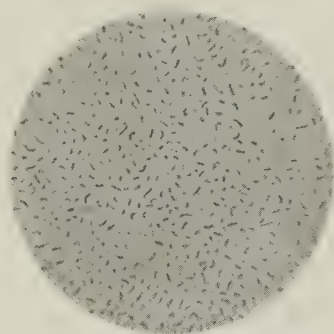


Fig. 5. Culture No. 1. Live preparation. Magnification 900x.

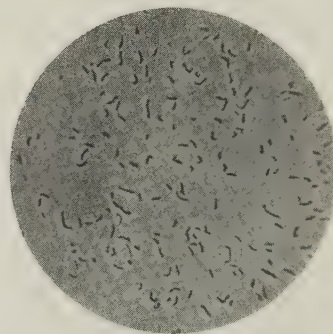


Fig. 6. Culture No. 3. Live preparation. Magnification 900x.

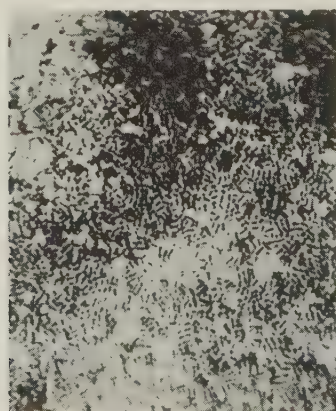


Fig. 7. Culture No. 21. Fixed preparation. Gentian violet stain. Magnification 900x.

Table 2. Utilization of Butyric Acid by Active Cultures in the Presence of Nonnitrogenous Organic Compounds (butyric acid in mg/100 ml of medium after six days of growth)

Culture No.	Sodium butyrate medium			Medium with sodium butyrate + mannitol or glucose		
	added	remained	utilized, %	added	remained	utilized, %
1	200	27.4	86.3	200*	27.4	86.3
3	183	18.9	89.8	183**	13.2	92.8
21	198	29.5	85.1	203*	36.0	87.2

*0.2% mannitol added in addition to butyrate; **0.2% glucose added in addition to butyrate.

Table 3. Butyric Acid Consumption by Active Cultures in Meat-Peptide Broth Containing 0.2% Potassium Butyrate (in mg per 100 ml of medium after six days of growth)

Culture No.	Butyric acid			Acetic acid	Reaction for NH_3
	added	remained	utilized, %		
1	234.1	Traces	100.0	0	+++
3	234.1	42.2	82.0	35.1	++
21	234.1	52.7	75.8	20.4	+

7.0–7.5. Can grow in the presence of increased concentrations (2.5–3.5%) of butyric acid salts.

From the description given, it follows that all three culture behave as ammonifiers; cultures Nos. 1 and 3 have a greater ammonifying capacity than culture No. 21.

3. The Utilization of Butyric Acid in the Presence of Other Carbon Sources

On the basis of the combination of characteristics found—good growth on meat-peptone media, ability to utilize carbohydrates, alcohols, and various mineral and organic nitrogen sources—the hypothesis was made that the utilization of butyric acid salts serves as a secondary biochemical function in the given cultures, becoming apparent only in the absence of more favorable carbon sources in the medium. In order to verify this hypothesis, several series of experiments were performed to determine the rate of utilization of butyrates in the presence of additional assimilable organic compounds.

a) Butyric acid consumption in the presence of non-nitrogenous organic compounds. The experiments were carried out in synthetic medium containing 0.2% sodium butyrate and 0.1% NH_4NO_3 . The following were used as additional carbon sources: 0.2% glucose for culture No. 3 and 0.2% mannitol for cultures Nos. 1 and 21. It is seen from Table 2 that the addition of mannitol or glucose did not reduce the rate of utilization of butyric acid at all.

Multiplication proceeded vigorously in all variants, with profuse accumulation of biomass on the bottoms of the vessels. In the variant with glucose, the qualitative reaction for sugar remained intense to the moment of almost complete disappearance of butyrate (after six days). Thus, butyric acid was selectively utilized by all three cultures in the presence of mannitol or glucose.

b) Butyric acid consumption in the presence of peptone. Inasmuch as the cultures grew well in meat-peptone media and behaved as ammonifiers on these media, the significance of butyric acid as a carbon

source could be shown most clearly in media containing peptone. For this purpose, the cultures were grown in ordinary meat-peptone broth containing 1% peptone and 0.2% potassium butyrate.

It is seen from Table 3 that under these conditions, all three cultures consumed butyric acid vigorously and that, as in the absence of butyrates, the capacity for ammonification was most strongly evident in cultures Nos. 1 and 3. The presence of small admixtures of acetic acid was usually observed in the products of distillation.

An entirely different picture was seen when the cultures were grown in synthetic medium containing 0.2% sodium butyrate and 0.2% peptone. It is seen from Fig. 8 that even after 11 days, about 60–70% of the initial amount of butyric acid remained unused in the medium. Since the utilization of butyrate was intensive in the same medium in the presence of NH_4NO_3 , the question of the influence of different forms of nitrogen on the given process arose. The results of these investigations will be given in another communication.

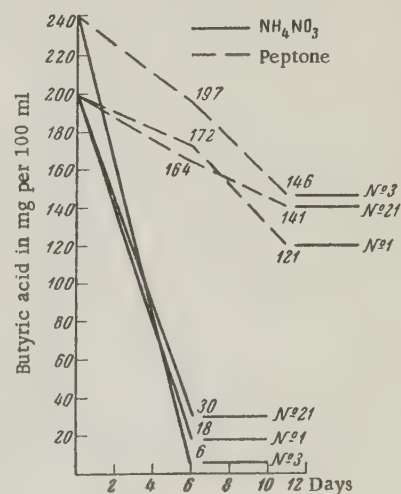


Fig. 8. Butyric acid consumption by active cultures in synthetic medium containing 0.2% peptone and 0.1% NH_4NO_3 .

The results obtained completely refute the hypothesis of the secondary significance of butyric acid in the carbon nutrition of the cultures isolated. The vigorous consumption of butyric acid salts by ammonifying bacteria in media as complete for them as meat-peptone broth makes it reasonable to consider this capacity as a specialized biochemical function of the given microorganisms.

The existence of microorganisms possessing the capacity to selectively utilize low-molecular fatty acids was, as is well known, discovered by Omelyanskii, who isolated a facultatively anaerobic coli-like bacterium (*Bact. formicum*) from horse manure which vigorously decomposes formic acid salts in peptone and meat-peptone media (Omelyanskii, 1903-1904). As for the selective utilization of butyric acid, it exists in various physiological groups among the anaerobes (methane-producing, sulfate-reducing). The presence of this function in the aerobes was first demonstrated by Werner (1933) who discovered, among a large number of butyrate-decomposing microorganisms which he isolated from soil, a group of new, sporogenous forms not previously described (*Bac. alpinus*, *Bac. segetalis*, *Bac. firmus*, *Bac. agrestis*), which consumed calcium butyrate actively in the presence of peptone and meat extract. Judging by their ability to liquefy meat-peptone gelatin and to produce ammonia on medium containing KNO_3 , these new microorganisms, as well as those described in the present communication, can also be related to the ammonifier group.

The discovery of specialized butyrate-decomposing aerobic ammonifiers is a good illustration of the long-

known variety of composition and heterogeneous character of the whole extensive group of so-called ammonifying bacteria, and points to the necessity for using the widest possible selection of specialized nutrient media in carrying out microbiological analyses of soil.

SUMMARY

1. A group of nonsporogenous aerobic ammonifying bacteria which have the capacity to utilize butyric acid salts selectively has been isolated from soil.

2. In many soil microorganisms, the capacity to utilize butyric acid salts is exhibited only in the presence of acetate in the medium.

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STUDY OF AMINO ACIDS RELEASED INTO THE MEDIUM BY MICROORGANISMS GROWING ON PETROLEUM WITH REFERENCE TO THE GENESIS OF OZOKERITE-LIKE BITUMENS

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At the present time, the ozokerite-like bitumens of the Minusinsk basin are regarded as formations which have arisen as the result of the processes of the transformation of petroleum rising to the surface along fissures in disrupted geological structures and dislocated strata.* Paraffin-base petroleum of the Bystryanskaya structure of the Minusinsk basin is a thick yellowish liquid and consists almost exclusively of hydrocarbons of the methane series, of different molecular weights and of normal and iso- structures (Sokolova, Mironov, and Nikitina, 1958). Ozokerite-like bitumens have a more or less viscous consistency and are encountered in the forms of individual small lumps and smears on rocks. Among different specimens, a transition is observed from bitumens of the type of light-colored yellowish paraffins to brown variants resembling humus substances. In the transition from the lighter, less altered varieties of bitumens to the more humified, an increase is observed in the content of oxygen, sulfur, and nitrogen, with a simultaneous decrease in the amount of carbon and hydrogen. Bitumens consist of a paraffin portion including fractions of light and dark paraffins, and a resinous portion which is the most metamorphized.

Many investigators believe that ozokerite-like bitumens were formed as the result of the microbiological oxidation of hydrocarbons emerging to the earth's surface along fissures (Barton, 1925; Hackford, 1932; Orlov, 1933; Davis, 1932, and others).

On the basis of experimental investigations, Uspenskii, Gorskaya, and Karpova (1947) think that the bitumen formations of the Minusinsk basin are products of the synthetic activity of microorganisms and are formed in the course of the anaerobic decomposition of petroleum hydrocarbons by desulfurizing bacteria. The authors used paraffin in their experiments. As has been noted in the literature, however, anaerobic conditions were not strictly observed in these experiments. Aside from this, according to our observations, paraffin-base petroleum is apparently unsuitable for the growth of sulfate-reducing bacteria.

On the basis of chemical investigations of the conversion products of paraffin-base petroleum and paraffin by the action of aerobic microorganisms, Mironov and Sokolova and co-workers (1957) came to the conclusion that the formation of ozokerite-like bitumens is associated with the activity of an aerobic microflora.

In their opinion, ozokerite-like bitumens are a complex mixture of the products of the synthetic activity of aerobic microorganisms as well as the products of the chemical oxidation of petroleum under aerobic conditions. It is pointed out that when cultures of aerobic microorganisms are grown on paraffin and on paraffin-base petroleum, products are formed which are enriched with oxygen, nitrogen, and sulfur and which resemble some varieties of natural bitumens in elementary composition. Investigations showed that compounds which in composition include amino acids are present in the ozokerite-like bitumens of the Minusinsk basin. Furthermore, the authors suggest that these compounds appear as the result of the decomposition of the cells of microorganisms growing on petroleum when it emerges to the surface of the earth.

In the present work, we undertook the task of determining the origin of these compounds. The demonstration of amino acids among the vital products of microorganisms growing on paraffin-base petroleum under aerobic conditions would make it possible to more closely approach the question of their origin in natural bitumens.

EXPERIMENTAL

Microorganisms were isolated from specimens of ozokerite-like bitumens from the Minusinsk basin on meat-peptone agar (MPA) and on mineral medium with paraffin-base petroleum as the source of organic material.

In order to determine the behavior of the isolated microorganisms toward paraffin-base petroleum, a liquid mineral medium of the following composition (in g) was used: Na_2HPO_4 -3.5; KH_2PO_4 -1.5; NaNO_3 -5; NaCl -10; FeSO_4 -0.02; CaCl_2 -0.04; Na_2SO_4 -0.04; tap water-1 liter; microelements according to Fedorov (Fedorov, 1951); pH-7.00. The only source of organic material was light-colored paraffin-base petroleum from the Bystryanskaya structure.

The medium was dispensed in 100-ml amounts into 250-ml Erlenmeyer flasks, and was sterilized and inoculated with a cell suspension (washings from a 2-day culture on MPA). After this, 2-3 ml of sterile petroleum was added to the flasks. A parallel control

*The indicated views do not pertain to the genesis of typical ozokerite deposits.

was set up: sterile medium with petroleum and mineral medium without added petroleum, inoculated with microorganisms also washed off from MPA. The cultures were kept in an incubator at 28° for six months.

At the end of the experiment, the culture fluid was filtered off from the bacteria and petroleum through a No. 3 membrane filter and analyzed qualitatively for the presence of amino acids by descending paper chromatography.

Parallel determinations for amino acids were made in culture fluid which had and had not previously been subjected to hydrolysis with 25% sulfuric acid, as has been employed in examining bitumens for amino acids (Sokolova et al., 1958).

Prior to chromatographic analysis, the culture fluid was freed of the mineral salts of the medium by a modified Baliga method (Baliga, Krishwamur, et al., 1955).

For this purpose, the liquid was evaporated to dryness, and organic substances were extracted from the residue with 2-3 ml of isopropyl alcohol. The entire solution was spotted on a single point on a strip of No. 2 (fast) chromatographic filter paper saturated with citrate-phosphate buffer at pH 4.00. A known mixture of amino acids was placed next to it. The strip of filter paper was passed three times through the solvent n-butanol-acetic acid-water (4:1:5) (Zaitseva and Belozerskii, 1957). The distilled water included in the composition of the solvent was also buffered to pH 4.00. Then, the chromatograms were dried in air and were sprayed with 0.1% ninhydrin solution in n-butanol or isatset† (Boyarkin, 1956). The chromatograms, dried in a current of air after spraying, were

kept in the dark for 24 hours. The spots were identified by the R_f value of the amino acids and by direct comparison with the known mixture of amino acids placed on the same strip of paper.

DISCUSSION

As has been pointed out, the basic problem of the work consisted in determining the amino acids in the products accumulating during the cultivation of microorganisms on medium containing paraffin-base petroleum under aerobic conditions. Amino acids were determined in cultures of the following microorganisms: Mycobacterium mucosum, Mycococcus ruber (two strains), Proactinium paraffinae, Proact. corallinus, Proact. actinoides, Penicillium notatum, and Pen. chrysogenum.

The analyses conducted showed that amino acids were found in the culture fluids of all microorganisms tested. Following hydrolysis of culture fluid, a large variety of amino acids was detected in a number of cultures. The amino acids in the medium are apparently in the form of hydrolyzable complexes. The weak color intensity of the amino acid spots revealed by the developers should be noted.

The following amino acids were found (Table 1). In culture fluid from Mycobact. mucosum: arginine, threonine, glutamic acid, alanine, methionine, leucine, and an unidentified acid located below proline; in cultures of mycococci: cystine, lysine, arginine, serine, glycine, threonine, alanine, proline, tyrosine, methi-

†I.e., isacet—transliteration of Russian, no further information being available—Publisher.

Table 1. Amino Acids Found in the Culture Fluids of Microorganisms on Paraffin-Base Petroleum Under Aerobic Conditions

Amino acids	Mycob. mucosum	Mycococ. ruber 18	Mycococ. ruber 6	Proact. corallinus	Proact. paraffinae	Proact. actinoides	Pen. notatum	Pen. chrysogenum	Control without inoculum
	hydr.*	hydr. w/h**	hydr. w/h	hydr. w/h young culture	hydr. w/h	hydr. w/h	hydr. w/h	hydr.	hydr. w/h
Cystine			+						
Lysine		+	+	+	+	+			
Histidine		+	+	+	+	+			
Arginine	+	+	+	+	+	+			+
Aspartic acid		+	+	+	+	+			
Serine		+	+	+	+	+			
Glycine		+	+	+	+	+			
Threonine	+	+		+	+	+			
Glutamic acid	+	+		+	+	+			
Alanine	+	+	+	+	+	+	+		
Proline		+	+	+	+	+			
Tyrosine		+	+	+	+	+	+		
Methionine		+	+	+	+	+			
Tryptophan				+	+	+			
β-Phenylalanine				+	+				
Isoleucine			+	+	+	+			+
Leucine	+	+	+	+	+	+		+	+
Spot below proline	+		+			+			
Yellow spot after norleucine		+	+		+	+		+	

*Hydr.—culture fluid subjected to hydrolysis; ** w/h—culture fluid not subjected to hydrolysis.

onine, isoleucine, leucine and an unidentified acid below proline; in culture fluid from *Proact. corallinus*, both subjected and not subjected to hydrolysis: lysine, histidine, arginine, aspartic acid, serine, glycine, threonine, glutamic acid, alanine, proline, methionine, β -phenylalanine, isoleucine, leucine; in culture fluid from *Proact. paraffinae*: lysine, arginine, aspartic acid, serine, glycine, threonine, glutamic acid, alanine, proline, tyrosine, methionine, isoleucine, leucine, tryptophan; in culture fluid from *Proact. actinoides*: cystine, lysine, serine, glycine, threonine, glutamic acid, alanine, proline, tyrosine, methionine, isoleucine, leucine, and an unidentified acid under proline; in the *Pen. notatum* culture: proline, tyrosine; in the culture fluid from *Pen. chrysogenum*: leucine.

If it is assumed that the production and accumulation of amino acids is associated with the greater or lesser ability of microorganisms to autolyze, the microorganisms investigated can be arranged in the following order. The most varied amino acid composition was found in *Proact. corallinus*. This organism, along with fungi, forms a thick pellicle on petroleum. The same amino acid composition, with the exception of histidine, tryptophan, and isoleucine, was also found in the unhydrolyzed culture fluid of a younger, two-month-old culture of the same microorganism (Table 1). Only very slight growth was observed and no amino acids were found in the culture fluid of *Proact. corallinus* on the same medium without the addition of petroleum. Quite a few different amino acids were also produced in the culture fluids of other proactinomyces. In the culture fluids of the mycobacteria and mycococci, the collection of amino acids was more limited. It should be noted that in the two strains of *Mycococ. ruber* tested, nearly the same amino acid composition was found in the culture fluids. This is probably explained by their belonging to the same species and by the identical conditions of cultivation of these microorganisms. The amino acid composition was the least varied in the fungus cultures. Even the amino acids which were found in the control without microorganisms—arginine, isoleucine, and leucine—were not found in the culture fluids of the fungi. This may be due to the consumption of these amino acids by the fungus.

Aside from this, the appearance of yellow spots located after norleucine was noted in many cases. These spots probably indicate the presence of pigments, the production of which has been observed in the culture fluid during growth of microorganisms on paraffin-base petroleum (Haas and Bushnell, 1944). On the chromatogram of the *Pen. chrysogenum* culture, several well-separated yellow spots after alanine could be distinguished.

It should be noted that, judging by the data in the literature, bitumens are enriched with amino acids in comparison with the original petroleum (Sokolova et al., 1958).

If the amino acid composition which we found in testing pure cultures of microorganisms growing aerobically on medium with petroleum is compared with the qualitative amino acid composition found in a specimen of bitumen (Mironov et al., 1957), the fol-

lowing becomes evident: All of the amino acids present in the bitumen were also found in the cultures of the microorganisms tested (Table 2). The amino acid

Table 2. Amino Acids Found in Culture Fluids of Microorganisms Growing Aerobically on Paraffin-Base Petroleum and in Natural Bitumen*

Amino acid	Encountered	
	in culture fluid of microorganisms growing on petroleum	in bitumen
Cystine	+	+
Lysine	+	+
Histidine	+	+
Arginine	+	-
Aspartic	+	+
Serine	+	
Glycine	+	+
Threonine	+	+
Glutamic	+	+
Alanine	+	+
Proline	+	
Tyrosine	+	
Methionine	+	
Tryptophan	+	+
β -Phenylalanine	+	
Isoleucine	+	
Leucine	+	+
Following proline	+	
Valine		+

*Data on the amino acid content of bitumen was taken from the work of Mironov and Sokolova and co-workers (1957).

composition in the culture fluids of pure cultures of microorganisms was more varied than in the bitumen. It should be noted that the amino acids in the bitumen were detected only after hydrolysis with 25% sulfuric acid and only in the more altered fractions of "dark paraffins".

The data which we obtained suggest that the process of enrichment of the original material by amino acids can occur under conditions of the aerobic utilization of paraffin-base petroleum by microorganisms. The amino acid-containing complexes found in bitumens apparently originated as the result of the death of microbial cells growing at the expense of petroleum, and are included in the composition of the bitumen in the form of the autolytic products of protein. The individual free amino acids can be further assimilated by other microorganisms.

SUMMARY

1. A large variety of amino acids which are probably products of autolysis of microbial cells was found in the culture fluid of all microorganisms tested growing on paraffin-base petroleum under aerobic conditions.
2. The similarity of the qualitative amino acid composition of ozokerite-like bitumens and of cultures of microorganisms growing on petroleum under aerobic conditions enables us to propose that the pathways of their formation are similar.

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[†]See English translation.

UTILIZATION BY MICROORGANISMS OF LOW CONCENTRATIONS OF ETHANE, DIFFUSING THROUGH SANDY-CLAY BLOCKS

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The study of the ability of hydrocarbon-oxidizing microorganisms to oxidize microquantities of gaseous hydrocarbons is of interest in determining the role of these bacteria in geochemical processes. The solution of this problem is also of significance in providing a basis for bacterial methods of exploration for petroleum (Mogilevskii, 1953).

The minimal concentrations of organic substances required for the normal activity of microorganisms has been determined frequently. The results obtained show large divergences among the investigations of various authors. Thus, according to the data of Hucker and Carpenter (1927), Fridlein (1928), Bigger (1937), and Penfold and Norris (1912), the concentrations of organic substances required for the multiplication of heterotrophic bacteria lie within the limits of 0.01 to 0.001%; according to Waksman and Carey (1935), Batterfield (1929), and Heukelekian and Heller (1940), they range from 0.005 to 0.001%. Zobell and Grant (1943) established that the presence of glucose in a concentration of 0.0001% is sufficient for the multiplication of marine bacteria, while according to Messineva's data (1958), an oligonitrophile of the genus *Pseudomonas* can grow using concentrations of organic material and nitrogen so small that they cannot be detected by the most sensitive methods of analytical chemistry. These divergences are possibly explained by differences in the materials studied and methods of quantitative counting of bacterial cells.

In evaluating the mentioned data in the literature, the very essential conclusion arrived at by N. V. and V. S. Butkevich (1936) should be kept in mind concerning the close relationship between limiting concentrations of organic substances required for growth of bacteria and temperature conditions and the chemical character of the compounds which serve as the sole carbon source. Under natural conditions, this relationship is apparently even more complex.

It should be noted that the investigations of all of the authors enumerated pertain to the limiting concentrations of organic substances which are in the dissolved state. An exception to this is Messineva's observation of the utilization of volatile organic substances.

As far as we know, the only extant data regarding minimal concentrations of gaseous carbon sources (methane 0.005% and propane 0.001-0.002%) required for the vital activity of microorganisms are in the investigations of Kuznetsov and his co-workers (1947).

We chose ethane for studying the ability of microorganisms to utilize low concentrations of gaseous

hydrocarbons. Ethane was chosen as the closest homolog of methane, since the formation and oxidation of methane by microorganisms has been relatively well studied. In this respect, ethane has hardly been examined to date, while the oxidation of small concentrations of it by microorganisms has not been studied at all. In the literature, there are only indications to the effect that ethane-oxidizing bacteria grow well in an atmosphere consisting of air and 30% ethane (Gubin, 1923; Bokova, 1954; Dostalek, 1954).

In our investigations, we endeavored to create conditions approximating natural ones as closely as possible. In nature, when gaseous hydrocarbons diffuse through sedimentary strata, constant concentrations are evidently never produced. The appropriate microorganisms must probably be adapted to conditions in which substances are constantly released into the medium in accordance with their utilization.

EXPERIMENTAL

In order to study the oxidation of hydrocarbon gases by microorganisms, a special device was constructed in which these gases entered, in known concentrations and by means of diffusion from a single source, into two experimental sandy-clay blocks, of which one contained live (experimental), while the other contained killed (control), hydrocarbon-oxidizing microorganisms. The ability of these microorganisms to oxidize ethane was established by simultaneous determination of its concentration in the space above both blocks. The device made it possible: 1) to simulate natural conditions to some extent, where in the presence of hydrocarbon gases, there is always a certain gradient in their concentration, and 2) to obtain identical initial concentrations in control and experimental blocks under experimental conditions.

The device was made of glass tubes connected by means of ground joints (Figure).

The collection of gas samples from tubes d and e to determine the concentrations of hydrocarbons which had diffused through the blocks was accomplished by means of displacing a specific volume of gas with salt solution through stopcocks f and g into the intake system of a TG5A gas analysis apparatus.

The concentrations of ethane above the blocks was determined two or three times during each experiment, most frequently after 10-20, 20-30, 30-40, or 40-50 days after the start of exposure.

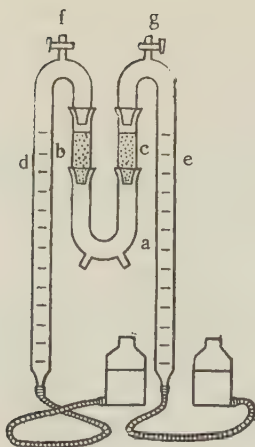


Diagram of device for determining hydrocarbon gases by bacteria. a) Tube filled with gas of known concentration; b) and c) tubes containing sandy-clay blocks; d) and e) graduated tubes for collecting gas which had diffused through the blocks.

In the present investigation, initial ethane concentrations of from 0.6% to $15 \cdot 10^{-4}\%$ were tested. However, in experiments with an initial ethane concentration of $15 \cdot 10^{-4}\%$, the concentration of gas decreased so much due to its sorption by the material of the blocks alone, that it could no longer be detected with the TG5A apparatus, the sensitivity of which is equal to $56 \cdot 10^{-5}\%$. Therefore, henceforth, results are given for experiments in which the initial ethane concentration was not lower than $28 \cdot 10^{-4}\%$.

The sandy-clay blocks were prepared from sterile sand (85%) and clay (15%) mixed under aseptic conditions and moistened with liquid mineral medium of the following composition (in %): NH_4Cl —0.1; MgSO_4 —0.02; K_2HPO_4 —0.08; KH_2PO_4 —0.02; NaCl —0.1; tap water; pH 7.2–7.5.

In preparing experimental blocks, live cells of ethane-oxidizing bacteria were previously added to this medium in known amounts, while for the preparation of control blocks, the same bacterial cells, but killed by boiling, were added.

Equal, weighed portions of the control and experimental mixtures were placed, one into compartment b and one into compartment c of the device.

In order to correctly evaluate the results obtained, we studied the process of diffusion of ethane through two control blocks of identical composition placed in tubes b and c of the device, repeating the experiment 16 times with different initial concentrations of ethane.

Determinations of ethane above the control blocks after various intervals of time while the temperature conditions of the diffusion fluctuated unequally showed that no substantial differences were noted in the ethane concentrations established above parallel blocks.

In the total number of determinations made (35), no divergence among the concentrations of ethane above the control blocks was noted in 88.6% of the cases, while fluctuations in ethane concentration not exceeding 8% were noted in 11.4% of the cases.

The results obtained can be regarded as satisfactory, and we considered it feasible to use the device constructed to set up experiments to determine the lower

limits of the concentrations of hydrocarbon gases at which their utilization by bacteria still occurs.

We tested a culture of *Mycobacterium perrugosum* var. *ethanicum*. Prior to setting up each experiment, the culture was transferred to liquid mineral medium of the indicated composition, and was kept in a gas mixture consisting of two parts of air and one part of ethane, until the appearance of visible signs of growth. The temperature conditions of the experiment varied. A total of 20 experiments lasting from 10 to 60 days was set up. The results are shown in Table 1.

As seen from the data presented, after passing through the block containing the culture of ethane-oxidizing bacteria, the amount of ethane decreased by half (or more) in comparison with the control in the overwhelming majority of cases (79%), and this process of ethane utilization continued up to 60 days.

At moderate (0.1–0.08%) and at low concentrations (0.006–0.0028%) of ethane, cases of 100% oxidation were observed (experiments 24, 27, 33, 34, 37). Cases of weak oxidation, when differences in ethane concentrations in the gas phase above the experimental and control blocks were within the limits of error of the measurement,* were few (10% as compared with 86% in control experiments).

The dependence of ethane utilization by bacteria on the initial ethane concentration is shown in Table 2.

It was found that the higher the concentration of ethane given, the greater was the absolute amount of ethane oxidized by the bacteria. However, this relationship was not direct, since when the ethane concentration was reduced eight times, the amount of ethane oxidized decreased only 2.7 times. When the concentration was further reduced 13 times, the amount of ethane oxidized decreased only 1.8 times.

It must also be noted that the diffusion of gas through the control blocks depends not only on the amount of ethane given but also on a number of other factors as well (temperature, pressure, moisture, etc.). This follows from the fact that when the concentration was altered by eight, and then by 13 more times, its content above the control blocks changed by 2.5 and 2 times, respectively.

The data presented make it possible to conclude that ethane-oxidizing bacteria are capable of carrying out the oxidation of ethane at all of its concentrations which we tested, as seen from Tables 1 and 2; moreover, at low concentrations, all of the ethane supplied was sometimes completely consumed.

In comparing our work with that of Kuznetsov et al. (1947), we should like to emphasize that these authors studied the oxidation of other gases (methane and propane), that these gases were drawn in a constant concentration through soil or liquid mineral medium containing the microorganisms, rather than diffusing through soil, as was the case in our work, and that the amount of methane or propane supplied to the culture per 24 hours was, at the lowest concentrations tested by the authors, twice as large as at the highest initial concentration in our experiments. In testing our lowest concentrations, the amount of ethane supplied to the bacterial culture per 24 hours was 236 times less than

*We designate the analytical results given below which were equal to $56 \cdot 10^{-6}\%$ or less with the letter L—the limit of sensitivity of the device—in order not to use zero, which would not be entirely valid.

Table 1. Comparative Changes in Ethane Concentration During Its Diffusion Through Control and Experimental Blocks.

Expt. No.	Initial C_2H_6 concentration under blocks, $\% \cdot 10^5$	Control Exptl.	C_2H_6 concentration above blocks ($\% \cdot 10^5$) at different exptl. durations (days)					Amount of C_2H_6 oxidized in $\%, \frac{C-E}{C} \cdot 100^{**}$			
			10-20	20-30	30-40	40-50	50-60				
18	65296	Control Exptl.	876 453	—	1003 453	1119 370	—	48.3	54.8	67.0	—
19	64624	Control Exptl.	749 181	843 181	798 268	—	—	75.0	52.3	63.5	—
20	64604	Control Exptl.	361 271	725 455	—	883 630	—	25.0	63.6	28.4	—
21	64512	Control Exptl.	723 272	811 388	1316 464	—	—	64.2	57.2	64.2	—
22	63280	Control Exptl.	2171 629	—	—	—	3133 1173	71.0	—	—	76.1
23	59880	Control Exptl.	826 390	1103 390	918 270	—	—	47.2	64.6	70.6	—
24	12600	Control Exptl.	896 728	448 L*	504 56	448 L	—	18.7	100	88.8	100
25	8120	Control Exptl.	280 56	336 168	—	—	—	80.0	50.0	—	—
26	8096	Control Exptl.	224 56	—	—	—	—	75.0	—	—	—
27	8040	Control Exptl.	448 336	280 56	336 L	—	—	25.0	80.0	100	—
28	7560	Control Exptl.	554 183	—	556 554	277 183	—	67.9	L	33.9	—
29	940	Control Exptl.	— —	85 85	423 170	339 L	—	L	59.8	100	—
30	940	Control Exptl.	— —	112 112	—	392 112	280 L	L	71.4	100	—
31	924	Control Exptl.	84 82	855 426	684 L	342 L	—	L	50.1	100	100
32	616	Control Exptl.	175 173	—	433 86	433 86	—	L	80.1	80.1	—
33	616	Control Exptl.	169 85	—	423 85	433 L	—	—	49.7	79.8	100
34	616	Control Exptl.	171 L	—	424 84	424 L	—	—	100	80.1	100
35	606	Control Exptl.	112 L	—	224 L	326 L	—	—	100	100	100
36	592	Control Exptl.	— —	342 265	428 159	428 79	—	—	22.5	62.8	81.5
37	280	Control Exptl.	56 L	—	112 L	—	112 L	—	L	100	100

*L—limit of sensitivity of device; **C—concentration of ethane above control block, E—concentration of ethane above experimental block.

Table 2. Changes in Intensity of Ethane Oxidation as a Function of Its Initial Concentration in the Experiment

Experiment No.	Avg initial ethane concentration under blocks in $\% \cdot 10^5$	Avg ethane concentration above control blocks in $\% \cdot 10^5$	Avg ethane concentration above exptl blocks in $\% \cdot 10^5$	Avg amount of ethane oxidized in $\% \cdot 10^5$
18,19,21,23	63579	794	324	470
25,26,27	8182	317	149	168
32,33,34,35	613	157	65	92

the minimal amount of propane in Kuznetsov's experiments.

Despite the differences stated, the basic patterns established by Kuznetsov and by us is the same: As the concentration of gaseous hydrocarbons received by the bacterial cultures is decreased, a reduction in the intensity of their oxidation occurs. However, this does not exhaust the conclusions which can be made from the material presented.

Due to the absence of determinations of the number of bacteria at the beginning and end of experiments, it cannot be directly concluded from our material whether or not their progressive multiplication takes place at the ethane concentrations tested (0.6–0.0028%). However, we could establish this by calculating the changes in intensity of ethane oxidation with time in each individual experiment and relating the values obtained to the entire mass of bacterial cells, rather than to a single cell as is usually done.

Such calculations were made for three experiments with the highest (0.6%, experiment 19), intermediate (0.08%, experiment 27), and low (experiment 37) initial concentrations of ethane.

Briefly, the method of calculation was as follows: using Clapeyron's equation in its calculation form, we determined the weight of ethane of the above-mentioned concentrations for the gas volumes in our experiments, and established what portion a gram-molecular weight of ethane it constituted. This made it possible to calculate the number of molecules above the control and experimental blocks in each experiment. The difference between these values, divided by the duration of the experiment, is equal to the number of ethane molecules oxidized by the entire mass of bacterial cells per 24 hours.

These calculations showed that at a high initial concentration of ethane (0.6%), the intensity of its oxidation remained at approximately the same level throughout the entire experiment (from 10 to 40 days). Consequently, no progressive multiplication of bacteria took place even at this concentration.

Comparing this result with the data of Dostalek (1954), who showed that, even at a high ethane concentration (30%), a culture of ethane-oxidizing bacteria passes into the phase of stationary growth after 10–12 days and the curve characterizing growth runs parallel to the axis of the abscissa, we come to the conclusion that the result which we obtained is explained by the fact that the determinations upon which the calculations of intensity of ethane oxidation are based pertain specifically to the stationary growth phase of the culture, because they were made on the 10–12th day from the start of the experiments.

Similar calculations for the intermediate concentration (0.08%) revealed some increase in intensity

of oxidation with time, while for the low concentration (0.0028%), it was even doubled.

These data enable us to propose that in the case of the intermediate and low concentrations, the phase of progressive growth is extended and, in making measurements after 10–35 days, we are still dealing with this phase.

Thus, ethane-oxidizing bacteria are capable not only of utilizing low concentrations of ethane as the sole source of energy, but also of progressive multiplication at such concentrations.

SUMMARY

1. Use was made of a specially constructed device allowing diffusion of ethane through sandy-clay blocks containing a culture of *Mycobacterium perrugosum* var. *ethanicum*, i.e., under conditions similar to "flowing cultures". It was shown by means of this device that ethane-oxidizing bacteria are able to oxidize ethane at concentrations ranging from 0.6% to 0.0028%.

2. With a decrease in the concentration of the diffusing gas, the rate of its utilization by bacteria diminishes.

3. The ethane-oxidizing bacteria are not only able to utilize the above low concentrations of ethane as the only energy source, but also reproduce progressively at these concentrations.

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GROWTH OF BACTERIA DURING CATHODE POLARIZATION OF STEEL IN SEA WATER

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Cathode polarization by superimposition of external current and by protectors* is widely employed for the protection of steel structures from corrosion in sea water. In connection with this, it is interesting to study the growth of bacteria on cathode surfaces. The development of three cultures of aerobic bacteria—*Bacillus megaterium*, *Pseudomonas fluorescens* var. *liquefaciens*, and *Bacillus mycoides*—is described below.

The experiments were conducted in flasks containing 0.25 liters of water from the Black Sea, using samples of steel St-3 measuring 25 cm² in area. The preparation of the samples, the inoculation of the experimental flasks with bacteria and their counting, as well as the equipment for polarization have been described earlier (Rozenberg, Ulanovskii, and Korovin, 1959). The results of the experiments are shown in Fig. 1.

Depending on the current density, the development of the bacteria went through two clearly defined stages. In the first stage, the number of bacteria rose sharply, reaching a maximum at a specific current density for each culture. Thus, for *B. mycoides* (curve 3), this maximum already set in at a current density of about 0.08 ma/cm², while for the other cultures, it was reached at about 0.2 ma/cm² (curves 1 and 2). In this case, the number of bacteria was many times greater than on control samples. After reaching the maximum, the development of bacteria started to weaken in the second stage. Thus, when the current density was increased from 0.08 to 2.5 ma/cm², the number of *B. mycoides* decreased tenfold.

On the cathode surface, OH⁻ ions accumulate continuously. At the same time, as the current density

increases, the pH of the near-cathodal layer also rises (Ulanovskii, 1956). It can be assumed that the basic factor determining the rate of bacterial growth in a given case is the pH value, which regulates the supply of nutrient substances to the cell (Fedorov, 1949) and in this manner influences the vital activity of the bacteria.

In actuality, as shown by experiments (Rozenberg, Ulanovskii, and Korovin, 1959), increasing the initial pH value from 7.5-7.6 to 9.0 markedly stimulated bacterial growth. The majority of bacteria usually reduce their growth rate at pH 9.0 (Fedorov, 1949; Mishustin, 1956). Whereas bacterial development goes on intensively in the first stage (depending on current density), and this is accompanied by an increase in pH to 9.0, their development decreases in the second stage, which is associated with a further increase in pH (greater than 9.0). *B. mycoides* is representative in this respect. Thus, the authors measured the pH on the cathode surface potentiometrically by means of a special device for collecting the near-cathodal layer of water (Ulanovskii, 1954); at a current density of 0.08 ma/cm², at which the activity of *B. mycoides* decreased sharply, the pH was about 10. However, different physiological groups of bacteria are not governed by this rule, and their growth decreases at a higher pH. Thus, for example, in denitrifiers this occurs at a pH of about 10-11 (Fedorov, 1949). As seen from Fig. 1, *P. fluorescens*, which is a denitrifier, has favorable conditions for growth at a higher pH. The viability of *P. fluorescens* starts to decrease simultaneously with that of *B. megaterium*. The latter was isolated by the authors from the water of the Black Sea, and it therefore cannot be more adapted to existence under conditions of a higher pH (the pH of the surface waters of the Black Sea is 8.3-8.45).

At low current densities of less than 0.005 ma/cm², cathode polarization checked bacterial growth; their numbers were somewhat less than the control. This is explained by the fact that at low current densities, there is oxygen depolarization, when oxygen brought to the steel surface by convection currents and diffusion is absorbed intensively (Tomashov, 1947). This is

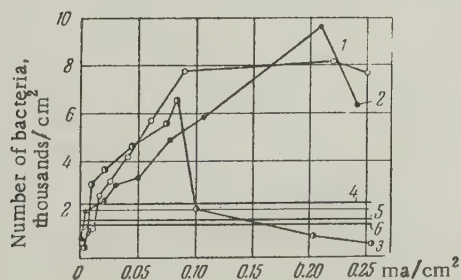


Fig. 1. Development of bacteria on a steel surface during cathode polarization. 1) *B. megaterium*; 2) *P. fluorescens*; 3) *B. mycoides*; 4), 5), 6) corresponding controls.

*Plate, anodal with respect to metal structure, by means of which the structure is protected from corrosion. For this purpose, a piece of metal (protector) having a lower electrode potential than the metal being protected is attached to the metal structure to be protected from corrosion. This produces a galvanic cell in which the protected structure itself serves as one electrode, the protector as the other electrode, and sea water as the electrolyte.

Table 1. Precipitation of Ca and Mg During the Processes of Denitrification and Ammonification

Culture	Bacteria, thousands/ml		Ca, mg/liter		Mg, mg/liter	
	at start	at end	at start	at end	at start	at end
<i>P. fluorescens</i>	0.75	5700	22.0	5.1	113.2	100.6
<i>B. mycoides</i>	0.55	1600	22.5	20.6	110.7	106.0

Table 2. Precipitation of Ca and Mg from Sea Water by Bacteria

Microorganisms	Sea water						Sea water + 0.005% peptone			
	bacteria, thousands/ml		Ca, mg/liter		Mg, mg/liter		bacteria, thousands/ml		Ca, mg/liter	
	at start	at end	at start	at end	at start	at end	at start	at end	at start	at end
<i>B. megaterium</i>	1.0	50.0	22.2	22.2	114.9	110.4	0.90	960.0	25.8	24.7
<i>P. fluorescens</i>	0.82	1930.0	22.2	21.3	116.1	110.4	0.79	2240.0	25.9	24.6
<i>B. mycoides</i>	0.65	20.0	22.8	21.3	116.8	111.5	0.72	60.0	25.8	24.6

unfavorable for bacteria with an aerobic type of respiration. Although the possibility of bacterial growth increases somewhat due to the slight increase in pH value occurring at these very low current densities, the viability of the bacteria is nevertheless depressed as the result of oxygen depolarization.

The influence of bacteria can take one of two directions: First, bacteria may increase protection from corrosion by supplementary precipitation of CaCO_3 and Mg(OH)_2 from sea water on the steel surface; second, bacteria may, on the contrary, decrease the protective effect of cathode polarization by increasing the rate of the autodissolution of steel. The influence of each of these factors increases or decreases depending on a number of causes.

Increasing Protection from Corrosion by Precipitation of Calciferous Films from Sea Water

As the result of electrochemical processes, calciferous films precipitate on the surface of steel during cathode polarization (Ulanovskii, 1956); such films have strong protective properties against corrosion. The basic components of these films are CaCO_3 and Mg(OH)_2 . It was important to show to what extent aerobic bacteria affect the precipitation of CaCO_3 and Mg(OH)_2 .

It is known that bacteria serve as one of the factors in the precipitation of CaCO_3 from natural solutions (Isachenko, 1948). Investigations show (Rozenberg, 1950) that CaCO_3 precipitation in sea water is quite substantial: As much as 17% of the total amount of Ca ions present in the solution precipitates in 5-6 days. The addition to sea water of organic matter in the form of sodium acetate and nitrogen in the form of $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 favors bacterial growth and, in connection with this, speeds up the sedimentation of Ca. The bacteriogenic precipitation of Mg occurs parallel to that of Ca.

In the first experiment, the intent was to determine in what ways *P. fluorescens* and *B. mycoides* exhibit, respectively, their denitrifying and ammonifying abilities. Therefore, sodium acetate was added to sea water, and KNO_3 was added for *P. fluorescens* and peptone for *B. mycoides*. After the ingredients were dissolved, the media were boiled for 10 minutes,

cooled, and were made up to original volume with distilled water; they were then shaken and filtered through a fluted paper filter. The media were dispensed in flasks and sterilized in an autoclave at 110° for 20 minutes. The experiment lasted for 14 days. It is seen from Table 1 that *P. fluorescens* caused considerable sedimentation of Ca. Its concentration in the solution decreased by 4.5 times. The precipitation of Ca by *B. mycoides* was less: about 10%. A considerable amount of Mg precipitated parallel with the Ca: about 12% in *P. fluorescens* and about 4% in *B. mycoides*.

The second experiment, set up for 10 days, was to have demonstrated to what extent CaCO_3 and Mg(OH)_2 precipitate due to the effect of bacteria in natural sea water and in water to which 0.005% peptone was added. The results of the experiment are given in Table 2.

In natural sea water, Mg precipitation was brought about by all of the cultures and constituted 4-5%. Ca precipitation was observed only in *P. fluorescens* and *B. mycoides* and was expressed by a value of 5-7%. The addition of 0.005% peptone to the sea water brought about the process of Ca precipitation in all three cultures. No precipitation of Mg was observed, however.

Decreases in the Protective Effect of Cathode Polarization

Experiments on the effect of bacteria on the corrosion of steel St-3 in sea water were carried out in liter flasks containing 400 ml of water. Weight losses of samples in sterile sea water and in water inoculated with the three bacteria under investigation are given in Table 3. All of these cultures increased the rate of corrosion considerably—by 10-30%. The effect of *B. mycoides* was somewhat greater than that of the other cultures. The effect of the bacteria on the corrosion rate decreased somewhat with time, although the number of bacteria rose continuously. The decrease in corrosion was probably associated with the precipitation of CaCO_3 and Mg(OH)_2 due to the effect of the bacteria on the cathodes of the microcouples which are responsible for the electrochemical corrosion of steel in sea water, because this precipitation becomes greater when the number of bacteria increases.

Experiments conducted with water from the Barents Sea also showed increased corrosion under the influ-

Table 3. Effect of Bacteria on the Corrosion of Steel St-3

Microorganisms	Interval of test, days					
	3		5		10	
	weight loss, g/m ²	number of bacteria per cm ²	weight loss, g/m ²	number of bacteria per cm ²	weight loss, g/m ²	number of bacteria per cm ²
Control	8.8	0	10.6	0	18.0	0
<i>B. megaterium</i>	10.4	30,000	12.8	45,000	19.5	219,000
<i>P. fluorescens</i>	10.2	48,000	12.0	65,000	19.9	300,000
<i>B. mycoides</i>	11.2	20,000	13.4	29,000	21.2	105,000

ence of the vital activity of putrefactive bacteria (Ulanovskii and Nikitina, 1956).

SUMMARY

1. The development of bacteria shows two distinct stages according to current density. During the first stage the number of bacteria greatly increases, attaining a maximum at a definite current density for each culture. In the second stage, linked with an increase in current density, the development of bacteria becomes less intense.

2. With increase in current density, the pH of the near-cathodal layer also increases. In the first stage, increased pH favors the development of bacteria; in the second stage, the pH values become unfavorable for development.

3. Bacteria may influence corrosion by: a) increasing protection from corrosion by the additional sedimentation of CaCO₃ and Mg(OH)₂ from sea water on the steel surface; b) decreasing the protective effect

of cathode polarization by increasing the rate of auto-dissolution of the steel.

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THE EFFECT OF MOLDS ON THE CORROSION OF METALS

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In investigating the effect of molds on various materials as well as on manufactured products (instruments, electrical machines, etc.) designated for use under conditions of moist tropical climate, we have frequently noted that the corrosion of current-carrying and other metallic parts is particularly intensive in those cases where the metals are in contact with mold growing on nonmetallic materials (plastics, varnished cambric, etc.).

The ability of fungi and other microorganisms to stimulate the corrosion of metals has been noted frequently in special literature pertaining to investigations of the microbial breakdown of organic materials in countries with moist tropical climates.

When Ganz and Wälchli (1955) were testing electronic instruments under conditions of moist tropics, they noted that the vital products of fungi growing on various materials frequently have an acid reaction and can cause the corrosion of metals or speed up the clouding of glass. Privezentsev, Maiofis, and Minina (1957), in describing the condition of cables following natural tests, found that the armoring tape of the cables becomes covered with rust and even breaks down as the result of the decomposition of nonantisepticized cotton casing of the cables (data of four-year tests). In cases where the outer coverings of the cables were antisepticized and, consequently, were resistant to microbial decomposition, the outward appearance and strength of the casing did not undergo any changes during five years.

The acid-producing capacity of various microorganisms, and particularly of molds, is commonly known. Under conditions where the fungi are adequately supplied with nutrients and oxygen from the air, they can synthesize and accumulate oxalic, citric, succinic, fumaric, shikonic,* gluconic, and other acids in the nutrient medium.

EXPERIMENTAL

Before going on to the investigation of the effect of the acids produced by fungi on the corrosion of metals, we set up an experiment necessary to confirm whether free acids actually accumulate on the surfaces of organic electrical insulation material during their fungal decomposition.

For this purpose, we selected a number of materials which differ in their resistance to fungal decomposition: highly destructible materials—brand LKh-2 varnished silk and varnished cambric on a cotton base; slightly decomposing melamine-glyptal-oil varnish, brand MGM-16; glass cloth and resistant materials—

polyisobutylene, Teflon and brand PKÉ-19 silicon organic enamel in the form of free films.

The samples of materials, measuring 50×50 mm, were inoculated with spore suspensions of the following species of fungi in distilled water: *Aspergillus niger* v. Tiegh., *A. amstelodami* (Mang.) Thom et Church, *Penicillium cyclopium* Westl., *Pen. brevicompactum* Thom, *Paecilomyces varioti* Bain, *Stachybotrys arta* Corda, *Chaetomium globosum* Kunze.

The species of fungi enumerated were suggested by the International Electrotechnical Commission for testing the resistance against the action of molds of materials and manufactured products (1954) designated for use under conditions of moist tropical climate, and are being used for this purpose by the majority of the scientific research organizations and factories in the Soviet Union.

The inoculated samples were placed in a chamber where a temperature of $30 \pm 2^\circ$ and 95–100% air humidity were constantly maintained.

In order to determine the amounts of free acids given off by the fungi during their growth on the samples of materials, the latter were placed in flasks containing 25 ml of 0.02 N Ba(OH)₂ solution. The flasks were tightly sealed with rubber stoppers and the barium hydroxide in the flasks was shaken slightly for three minutes. After this, the barium hydroxide was titrated with 0.02 NHCl in the presence of phenolphthalein. Samples of materials which were exactly the same but which had not been inoculated with fungi served as controls.

The results of determinations of the amount of acids produced on the surface of the samples of materials given in Table 1 show that a considerable amount of free acids was produced on brand LKh-2 varnished cambric and varnished silk, which were severely affected by the fungi. Moreover, as the time during which the fungi acted on LKh-2 varnished cambric was increased, the amount of acids on samples of it increased as well.

The fungi grew poorly on samples of MGM-16 varnish, and less acid was found on them than on the very moldy varnished cambric samples.

No acid production was found on samples of materials resistant to fungal decomposition (polyisobutylene, Teflon, and PKÉ-19 enamel).

These data show that when organic electrical insulation varnishes and varnished cambrics are decomposed

*Transliteration of Russian, no further information being available—Publisher.

Table 1. Results of Determinations of Amount of Acids Produced on the Surface of Samples of Materials Being Tested for Their Resistance to Decomposition by Molds

Materials and duration of test	Amount of acids per 100 cm ² of sample surface of materials, meq	Intensity of fungal growth on samples
LKh-2 varnished cambric (21 days)	0.157	Good growth
" " " 7.5 months	0.383	" "
Varnished silk (21 days)	0.104	" "
MGM-16 varnish (21 days)	0.032	Poor growth
Teflon-4 (28 days)	0	No growth
Polyisobutylene (28 days)	0	" "
PKÉ-19 enamel (28 days)	0	" "

Table 2. Results of Determinations of Acid-Producing Capacity of Fungi on Nutrient Medium with Various Contents of Glucose

Fungi	Glucose concentration, %	Thickness of yellow layer of medium in % of total thickness of medium layer							
		duration of incubation, days							
		3	4	8	11	13	16	20	30
<i>Asp. niger</i>	3	17	27	100	100	100	100	100	100
	5	24	46	100	100	100	100	100	100
	10	33	61	100	100	100	100	100	100
<i>Asp. amstelodami</i>	3	0	0	49	69	17	0	0	0
	5	0	21	95	95	100	29	0	0
	10	5	29	100	100	100	100	100	100
<i>Pen. cyclopium</i>	3	13	36	100	78	78	0	0	0
	5	23	44	100	100	100	100	100	0
	10	32	67	100	100	100	100	100	100
<i>Pen. brevi-compactum</i>	3	0	0	0	0	0	0	0	0
	5	5	31	100	100	100	0	0	0
	10	22	47	100	100	100	100	100	100
<i>Paec. varioti</i>	3	0	0	30	62	63	0	0	0
	5	0	0	75	95	100	100	100	0
	10	0	0	87	100	100	100	100	100

Note. Two species of fungi—*S. atra* and *C. globosum*—had no acid-producing capacity under the given experimental conditions.

by fungi, free acids accumulate on their surface, and that the amount of these depends on the intensity of growth of the fungi on the materials, i.e., on the intensity of the process of decomposition of the materials.

Resistance to fungal decomposition of polyester resins used as electrical insulation varnishes decreases markedly when vegetable oils are introduced into their composition (Brown, 1946; Al'bitskaya and Shoposhnikov, 1959).

During investigations of the causes of the spoilage of plant oils, particularly of palm and coconut oils, it was established that the decomposition of the oils is connected with oxidative breakdown caused by molds. The fatty acids of the oils are first released by the action of lipolytic enzymes, and are then decomposed by fungi with the production of a large number of products, including free acids, which accumulate in the medium (Foster, 1950).

Taking this into consideration, it can be assumed that the production of acids on the surface of samples of varnished cambrics and MGM-16 varnish are due to the decomposition of vegetable oils included in the composition of the given materials.

The acid-forming capacity of fungi varies greatly, depending on the species and even the strain of the fungus, as well as on the method of its cultivation and the composition of the nutrient medium. Fungi of the genera *Aspergillus* (particularly *A. niger*) and *Penicillium* are among the most active acid producers (Lilly and Barnett, 1953).

In order to investigate the acid-producing capacity of the seven species of fungi enumerated which were used for testing the resistance of nonmetallic materials to decomposition by fungi, we used a method similar to that proposed by Davis (1948).[†] A nutrient medium promoting maximal acid production by these species was chosen at the same time.

The fungi were grown on Czapek's medium, in which the amount of glucose was varied from 3 to 10%. In order to be able to follow changes in the reaction of the medium without interrupting the experiment, brom cresol purple was added to it as an indicator. In alkaline medium (pH greater than 6.8), brom cresol purple has a blue-purple color, while in acid medium (pH less than 5.2), it is yellow. The nutrient medium to which the given indicator is added takes on the corresponding color as well.

Fungi which are capable of acid production, when grown on the surface of nutrient agar medium, cause the yellowing of its upper layers under the mycelial film. The yellow color gradually spreads downward into the mass of the medium. We took the rate of yellowing of the medium and the thickness of the yellow layer as indicators of the acid-producing activity of the fungi.

In the experiment described, the nutrient medium (pH 6.8) was dispensed in 5-ml amounts in test tubes

[†] Cited according to Foster (1950).

(internal diameter 15 mm). The test tubes were placed absolutely vertically. After the medium solidified, its surface was inoculated with the spores of pure cultures of fungi, and after the colonies started to grow, regular observations were made of the acidification of the nutrient medium by measuring the thickness of the layer which had become yellow. Observations were made for 30 days. Later, the medium started to dry out rapidly, and measurements were stopped.

The results given in Table 2 show that only two species of fungi—*S. atra* and *C. globosum*—had no acid-producing capacity under the given conditions. The rest of the species brought about the acidification of the medium to a greater or lesser extent, depending on the glucose content of the medium.

A. niger had the strongest acid-producing capacity: As early as the eighth day of the experiment, the entire thickness of medium in the test tubes containing this culture had already acquired an intense yellow color; the pH of the medium, measured on the 45th day after the start of the experiment, dropped to 3.1–3.5 in the medium with 3% glucose, and to 2.1–2.3 in the medium with 10% glucose. It was noted that increasing the glucose content of the medium promoted more intensive and prolonged acid production. Specifically, when the glucose content of the medium was 3%, *A. amstelodami*, *Pen. cyclopium*, and *Paec. varioti* caused acidification of the medium for a short period of time; after the thickness of the yellow layer of medium in the test tubes containing these cultures reached a maximum, a purple color again appeared in the upper layers of the medium, and gradually spread downward from the mycelial pellicle through the entire thickness of the medium. This indicates that the acids synthesized by these fungi cannot accumulate in medium containing 3% glucose. *Pen. brevi-compactum* showed no noticeable ability to acidify the given medium: The medium in tubes containing a culture of this fungus retained its purple color throughout the entire experiment.

On medium containing 10% glucose, all of these species caused rapid and long-lasting yellowing of the entire layer of medium. No secondary appearance of the purple color was noted in any of the variants containing this medium during 30 days. Therefore, further investigations of the effect of the vital products of fungi on the corrosion of metals were carried out with nutrient medium containing 10% glucose.

Copper (diameter 0.070 mm), oxidized aluminum (diameter 0.420 mm), and steel (diameter 0.415 mm) wires were selected for the investigations.

Nutrient agar medium was dispensed in 200-ml amounts in 350-ml Erlenmeyer flasks and was inoculated with an aqueous spore suspension of pure cultures of the above-mentioned species of molds. Sterile samples of wire measuring 8 cm in length were placed in the flasks on the surface of the growing mycelium 2–4 days after inoculation and were gradually overgrown by the mycelial film from all sides. Identical samples placed on the surface of sterile nutrient medium served as controls. The experiments lasted for 28 days. The temperature was maintained at a level of $30 \pm 2^\circ$ for all variants.

After 28 days, the samples were removed from the flasks and their breaking strength was determined.

It should be noted that when the wire samples were being placed in some of the experimental variants, it

was impossible to lay them down strictly parallel to the surface of the medium; the samples sometimes became partially submerged in the medium, which led to the retardation of their corrosion due to the inadequate access of oxygen from the air; sometimes, on the contrary, the samples lifted up above the surface of the medium, in which cases some portions had no contact with the fungal film, which could also distort the results of the experiment. Therefore, in determining breaking strength, we got considerable scattering of the data in a number of variants. Taking this into consideration, we decided that it was not enough to give only average data; in Tables 3, 4, and 5, we present the results of breaking strength determinations of all of the samples tested so that the maximal and minimal values can be seen.

Samples of steel wire underwent the greatest alterations as compared with the control (Table 3). In variants with a pure culture of *A. niger*, the samples of steel were transformed into formless accumulations of oxides in 28 days, and it was impossible to separate them from the mycelial film of the fungus. In variants with cultures of *A. amstelodami* and *Pen. cyclopium*, all of the samples fell apart into small pieces. The same was observed in the two duplicates of the experiment with the *Pen. brevi-compactum* culture. The breaking strength of steel wire which was in contact with the *Paec. varioti* culture also decreased sharply. The samples of steel tested in flasks of *S. atra* and *C. globosum* cultures underwent no noticeable alteration as compared with samples which were on the surface of sterile nutrient medium.

Copper wire proved to be more resistant to the action of fungal metabolites (Table 4). It was observed to fall apart into separate pieces in only one repetition of the experiment with the *Pen. cyclopium* culture. Marked reduction in the breaking strength of copper wire also occurred due to the action of the *A. niger* culture: Its strength decreased by an average of 80% in comparison with the control. The strength of copper wire also decreased considerably under the influence of the *Pen. brevi-compactum*, *A. amstelodami*, and *Paec. varioti* cultures. No noticeable reduction in the strength of copper wire was noted in the experiments with cultures of *S. atra* and *C. globosum*.

The same thing was also observed in the experiment with oxidized aluminum wire when performed with these cultures (Table 5).

Oxidized aluminum was found to be the most resistant to the action of the vital products of molds. The falling

Table 3. Results of Determinations of Breaking Strength of Steel Wire

Fungi	Breaking strength, kg				
	repetitions of experiment				average
	1	2	3	4	
<i>Asp. niger</i>	0	0	0	0	0
<i>Asp. amstelodami</i>	0	0	0	0	0
<i>Pen. cyclopium</i>	0	0	0	0	0
<i>Pen. brevi-compactum</i>	0	0	7.4	3.6	2.8
<i>Paec. varioti</i>	1.7	9.0	10.4	—	7.3
<i>S. atra</i>	25.6	25.4	28.0	25.2	26.1
<i>C. globosum</i>	21.2	29.4	29.3	31.0	27.7
Control (sterile medium)	23.0	32.0	27.0	30.0	28.0
Original samples	34.0	34.0	33.8	33.7	33.9

Table 4. Results of Determinations of Breaking Strength of Copper Wire After It Had Been in Contact with Pure Cultures of Molds

Fungi	Breaking strength, g				
	1	2	3	4	average
<i>Asp. niger</i>	20	23	24	—	22
<i>Asp. amstelodami</i>	84	42	46	82	64
<i>Pen. cyclopium</i>	0	56	55	—	37
<i>Pen. brevi-compactum</i>	66	74	39	—	60
<i>Paec. varioti</i>	54	54	54	50	53
<i>S. atra</i>	104	79	109	110	100
<i>Ch. globosum</i>	105	102	102	—	103
Control (sterile medium)	106	105	105	110	107
Original samples	185	185	189	181	185

Table 5. Results of Determinations of Breaking Strength of Oxidized Aluminum Wire After It Had Been in Contact with Pure Cultures of Molds

Fungi	Breaking strength, g				
	1	2	3	4	average
<i>Asp. niger</i>	0	0	945	—	—
<i>Asp. amstelodami</i>	850	1030	1330	1120	1095
<i>Pen. cyclopium</i>	1170	1160	1260	1160	1187
<i>Pen. brevi-compactum</i>	1270	1240	1290	1170	1242
<i>Paec. varioti</i>	1310	1300	1140	1305	1264
<i>S. atra</i>	1370	1270	1370	1380	1348
<i>Ch. globosum</i>	1350	1370	1370	1370	1365
Control (sterile medium)	1370	1370	1370	—	1370
Original samples	1390	1390	1390	—	1390

apart of occasional samples was observed only in the experiment with the *A. niger* culture. Some reduction in the breaking strength of the wire in comparison with the control was also noted in experiments using cultures of *A. amstelodami*, *Pen. cyclopium*, *Pen. brevi-compactum*, and *Paec. varioti*.

When samples of oxidized aluminum were examined, a considerable decrease in diameter was noted in those samples which had been in the films of the *A. niger*, *A. amstelodami*, *Pen. cyclopium*, *Pen. brevi-compactum*, and *Paec. varioti* cultures. Their diameters decreased to 0.355–0.365 mm on the average. Moreover, no such decrease in the thickness of the samples was observed on portions of the wire which had been above the surface of the film. Consequently, the breakdown of oxidized aluminum occurred chiefly within the mass of the fungal film. Naturally, with such a decrease in the thickness of the wire, the oxide layer present on the surface of the samples was very rapidly disrupted.

The data obtained show that the corrosion of steel, copper, and oxidized aluminum was the most intensively manifested in experimental setups with cultures of *A. niger*, *A. amstelodami*, *Pen. cyclopium*, *Pen. brevi-compactum*, and *Paec. varioti*, i.e., with those cultures which we had noted as active acid producers. The intensity of the corrosion of metals in experiments with cultures of *S. atra* and *C. globosum*, which do not cause acidification of the medium, was practically indistinguishable from the intensity of corrosion in control flasks containing sterile nutrient medium. This proves that the acids given off by fungi into the surrounding nutrient medium cause the corrosion of metals.

SUMMARY

1. Molds produce acids when growing on organic electrical insulation varnishes and varnished cambrics.
2. Of the fungal species tested, *Aspergillus niger*, *A. amstelodami*, *Penicillium cyclopium*, *Pen. brevi-compactum*, and *Paecilomyces varioti* are capable of producing acids.
3. The breaking strength of samples of steel, copper, and oxidized aluminum wire decreased considerably under the influence of the acids given off by the fungi, and the samples were completely destroyed in a number of cases.

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THE USE OF THE DIRECT-COUNT METHOD FOR THE MICROBIOLOGICAL ANALYSIS OF PURIFICATION OPERATIONS AT A WATERWORKS

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The principal aim of such water-purification measures as sedimentation, coagulation, and filtration is to eliminate suspended impurities, including both coarse material (sand, phyto- and zooplankton, plant and animal debris) and the fine particles measured in microns or fractions thereof (silt particles, bacterial cells, and colloids).

The water-purification operations at water-supply stations are made effective by frequent and routine control inspections by means of physicochemical, hydrobiological, and bacteriological test methods, with the ultimate goal of producing potable water in conformity with requirements of the State Standard. Up to now there has been no functional division of this sanitary inspection between the technical evaluation of the treatment plant and the hygienic assay of the quality of the treated water.

Aside from that, control test methods in use at the present time cannot be regarded as adequate. Thus, identification of suspensions in the water by means of visual or weight methods for determining turbidity and clearness does not give a picture of the nature of these suspensions (qualitative composition) or of the effect of the treatment.

Bacteriological analyses ought to provide the most valuable and significant data on sanitation. When these tests are carried out in accordance with the All-Union State Standard [GOST-5216-50(55)], however, they are both laborious and time-consuming, requiring 24-48 hours. In practice this can be cut down to 4-6 hours, but even under these circumstances results of the analyses cannot be examined in time to be utilized effectively. When treatment includes disinfection of the water, and preliminary chlorination in particular, these findings, as will be shown later, are of use in assessment of this bactericidal activity alone, and do not measure the effect of the other purification processes.

Out of these considerations we took up Razumov's proposal to use his direct-count method of water microscopy (Razumov, 1932, 1953, 1957) for the purposes of technical control tests on sanitation operations in a waterworks plant, introducing several modifications in order to reduce and simplify his procedure.

METHODS

In carrying out our investigations, we made several alterations in Razumov's technique, which would enable

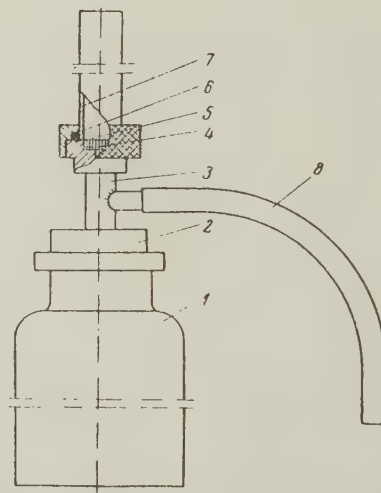


Fig. 1. Dolgov-Razumov filter apparatus. 1) Glass receptacle (manufactured item); 2) rubber stopper (manufactured, to fit receptacle); 3) brass pipe; 4) pumice pad, 10 x 3 mm; 5) hollow brass screw; 6) rubber washer; 7) glass pipe; 8) rubber hose, length ~ 200 mm.

us to cut down analysis time to 15 minutes and to minimize the labor required.

The altered method consists in the following: The sample volume (5-30 ml) of water to be examined (depending on bacterial proliferation) is passed through a No. 3 membrane filter in a Dolgov-Razumov apparatus, which has a filter surface 10 mm in diameter (Fig. 1). The filter with the recovered bacteria is then dried in a thermostatically controlled chamber and stained for five minutes on filter paper moistened with a 3% carbol fuchsin solution. The excess color is removed by blotting the filter five or six times on filter paper moistened with distilled water. The filter is again dried and the stain completed after 1.5 minutes on filter paper moistened with a 1% aqueous solution of methylene blue, according to Zuikova's method (1959).

The filter preparation is decolorized to a light blue by blotting it five or six times on filter paper moistened with water; it is then dried and placed in a drop of immersion oil on a glass slide. This clarifies the preparation, after which another drop of immersion oil is placed on its surface for microscopy at 1350x.

Quantitative determination of the bacteria is accomplished with the aid of a counting chamber, which di-

vides the microscopic field into equal squares of a predetermined surface area.

The total number of bacteria in 1 ml of water is calculated by the formula:

$$X = \frac{S \cdot n}{s \cdot v}$$

in which X represents the number of bacteria in 1 ml of water; S —the predetermined surface area of one square (μ^2); s —the total surface area of the filter (μ^2); n —the number of bacteria in one square of the counting chamber; and v —the volume of filtered water in ml.

The number $\bar{n}$ is the arithmetic mean for 50 computations of the number of bacteria in a square of the counting chamber.

The Purification Program at the Northern Waterworks Plant

Raw water fed into the plant from the reservoir first undergoes preliminary chlorination. A coagulant solution (aluminum sulfate) is then added. After mingling in a mechanical agitator this mixture is fed into the settling tank. Here the coagulant forms clumps of $Al(OH)_3$, which attract the greater part of the contaminating material as they settle out in the basin. From the settling basin the water passes to filters. The effluent yielded here is chlorinated for the second time in the contact tank as a further disinfection measure, and is then stored in clear-water reservoirs, from which it will be pumped up again to the distribution network.

EXPERIMENTAL

At every stage in the treatment process, in all seasons of the year, we examined the water by means of two test methods: the standard (Koch) and the "direct".

Inasmuch as preliminary chlorination is a regular procedure at the Northern Plant, a substantial number of bacteria are destroyed in the first step, and in the following steps Koch's method reveals only isolated cells of saprophytes. With the standard method, then, it is only the chlorination process which is being tested. This method cannot give an evaluation of the subsequent processes.

Parallel analyses of this same water performed by the "direct" method illustrate to what extent the water is freed of bacteria during subsequent stages in the treatment (Table 1).

Assessment of the Effectiveness of the Settling Basin

The effectiveness of a settling basin depends upon: the quality of the influent, the dosage of coagulant, the time when settling takes place, the rate of water movement, the length of the operation cycle of the settling tank, and, consequently, the quantity of sediment which accumulates in it. The "direct" method enabled us to establish that when the correct technical conditions

are observed, a settling basin can retain up to 75% of the bacteria from the water which passes through it. Figure 2 shows that in 1958, water entering the settling basin (raw) had an over-all bacterial count which fluctuated within the extremes of 33,000 to 2,000,000/ml, while the water leaving it contained from 20,000 to 900,000 cells/ml. The percentage of bacterial precipitation in the settling tank fluctuated, but on the average it was about 42%.

In Fig. 2 it can also be seen that on some days there occurred a "leakage" of bacteria from the settling basin, i.e., there were more bacteria in the water leaving the basin than in the water entering it.

A careful analysis of all conditions in the settling basin revealed the cause of this phenomenon, and in this way it served to demonstrate the applicability of the "direct" method in uncovering variations in the operations of a given plant which cannot be detected by physicochemical and standard bacteriological methods.

Thus, on April 28, water entered the basin containing 96,000 bacteria per ml; on leaving, it contained 120,000 cells/ml. It happened that the operational cycle of the basin had taken 70 days, whereas under normal conditions the cycle was allotted 40 days; a large quantity of sediment had thus accumulated, and the rate of water movement increased from 8.2 to 13 mm/sec; the settling time was cut down from 2.3 to 1.5 hours.

Later, on July 9, the operational cycle of the settling basin took 60 days, a large quantity of sediment had accumulated, the rate of water movement had increased from 7.5 to 9.7 mm/sec, and the settling time was cut from 2.6 to 2.0 hours. The amount of coagulant dropped from 30 to 18 mg/liter, which had a negative effect on flocculation activity and further restricted the process of bacterial precipitation.

All these disturbances in the operation of the settling tank led not only to the increase in bacterial count from 33,000 to 52,000 cells/ml, but also to increased turbidity and coloration of the water after sedimentation.

On the other dates of bacterial "leakage" (July 18, 21, and 26) the operating conditions of the settling basin remained the same as on July 9, but the dosage of coagulant was accordingly increased to 32.41 and 35 mg/liter. Color and turbidity of the water leaving the settling tank at that time were normal, but a "leakage" of bacteria was observed; thus, the bacterial count correspondingly increased from 60,000, 64,000, 88,000, and 108,000 cells/ml to 96,000, 88,000, and 108,000 cells/ml (Fig. 2). A detailed analysis of these events enabled us to establish the causes for this bacterial "leakage".

On October 18, the rate of water movement had increased from 8.2 to 10.8 mm/sec; the settling time was cut from 2.3 to 1.8 hours. Color and turbidity of the water at this time were almost unchanged, while the bacterial count had risen from 156,000 to 240,000 cells/ml.

On December 25, the rate of water movement increased from 9.1 to 13.9 mm/sec and the settling time was cut from 2.1 to 1.4 hours. The dosage of coagulant was low—18 mg/liter. Turbidity and color of the treated water showed a minor increase at this time, while the bacterial count had risen from 84,000 cells/ml (in the raw water) to 544,000 on leaving the basin.

From these examples it is evident that the findings of the "direct" method are more sensitive indicators

Table 1. Average Number of Bacteria in 1 ml of Water as Treatment at the Plant Proceeds

Test sample	Standard method	"Direct" method
Raw water	100	185,000
Settled water	2	130,000
Filtered water	1	82,000
Fully treated water	2	45,000

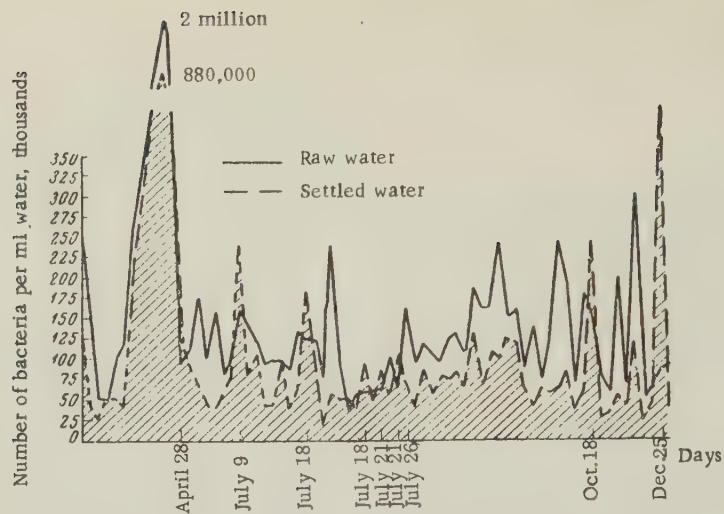


Fig. 2. Bacterial precipitation in the settling basin.

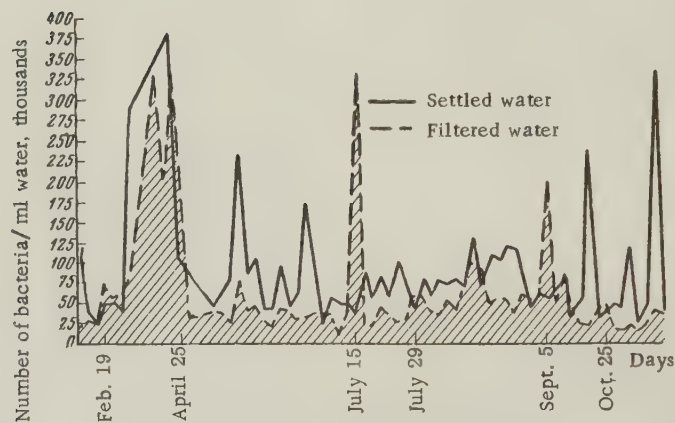


Fig. 3. Retention of bacteria by the filters.

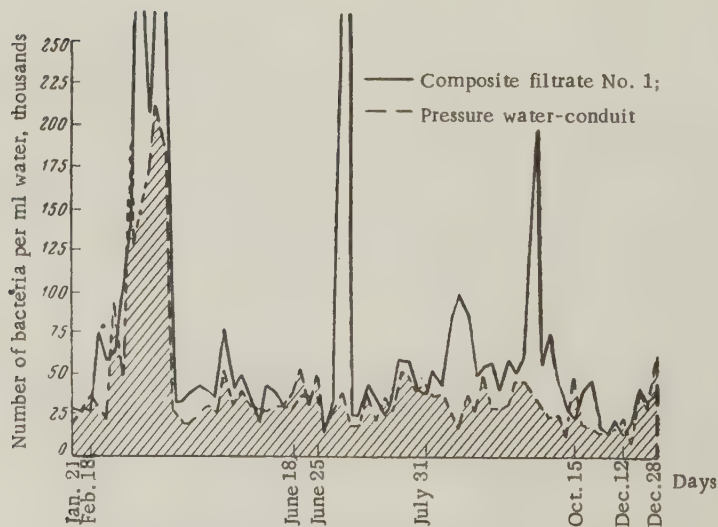


Fig. 4. Reduction of bacterial count in the reservoirs.

of the effectiveness of the settling basin operation than are results of physicochemical analyses and quantitative determination of bacteria by Koch's method.

Assessment of the Effectiveness of the Rapid Filter Operation

From the settling basin, the water passes to filters for removal of the remaining impurities.

For the present work we analyzed a composite filtrate which had been collected from filters of various types. The results of this series of tests, which were carried out by the "direct-count" method, are presented in Fig. 3, where it can be seen that in water entering the filters, the bacterial count fluctuated from 20,000 to 900,000 cells/ml, while in the filtrate it remained at 30,000-50,000 cells/ml.

Thus, on this occasion, the filters retained up to 94% of the bacterial cells in the water, with an average retention of 47%.

In addition to this, it is obvious that here, too, bacterial "leakage" was occurring. Thus, on July 15, water entered the filters with a content of 52,000 cells/ml, while in the filtrate, the number had risen to 1,620,000 cells/ml.

It happened that at that time the speed of the filtering operation had changed from 5.8 to 6.8 m/hour. Along with this, turbidity increased from 1.6 to 1.9 mg/liter and color from 15 to 20°.

In another case of bacterial "leakage" from the filters (July 29), water entered the filters containing 40,000 cells/ml, while on leaving the filters it contained 60,000 cells/ml. This occurred as a result of the acceleration in the speed of the filtration process from 6.4 to 7.2 m/hour. Turbidity and color in this case showed a minor increase. Bacterial "leakages" from the filters occurred on the following days as well, but it was impossible to find an explanation related to the mechanics of the filtering operation since filtrate No. 1 (which was analyzed in this work) had been obtained from filters of various types ("AKKh," double level, and the usual rapid filter), which differed in capacity, degree of contamination, filtration rate, length of filtration cycle, and other characteristics.

Assay of the Quality of the Water in the Storage Reservoirs

After the second chlorination the water passes from the contact tanks to storage reservoirs of clear water, from which it will be fed to the distribution network.

At the time when this work was carried out, water stood for an average of 6 to 8 hours with a presence of 0.33 mg/liter of residual chlorine in the storage reservoirs.

Under these conditions there will occur some sedimentation and a settling of bacteria on the floor and walls of the tank, as well as lysis of the bacteria killed by chlorine.

As illustrated in Fig. 4, the "direct-count" method plainly shows that on some occasions the bacterial count in the reservoir dropped to 84%, with an average of 33%.

This cannot be detected with the standard method. Using Koch's method (GOST 5216-50) we can establish that the water in the reservoir is not sterile; single bacterial cells are revealed, at 1/ml, which have survived chlorination and have become adapted to the amount of residual chlorine present.

Sharp changes in these conditions promptly bring about a "leakage" of bacteria from the reservoirs into the water-distributing network. These changes can come about as a result of 1) disruption of the contact reservoir operation and a sharp reduction in the chlorine dosage which causes disinfection to be incomplete, or 2) fluctuations of the water level in the tanks, uncovering the walls and creating favorable conditions (no residual chlorine) for the multiplication of bacteria which have settled there; moreover, under these circumstances bacteria may automatically be washed from the tank walls with the water movement, whereas this does not take place when a steady water level is maintained.

Cases of bacterial "leakage" from the storage reservoirs are shown in Fig. 4. These "leakages" were caused by the sharp fluctuations of the water level in the tanks (Table 2).

Table 2. The Effect of Fluctuations of the Water Level in the Storage Reservoirs on Bacterial "Leakage" (1958)

Date	No. of reservoirs	Water level at midnight, in meters	Water level at 8:00 A.M., m	Change of water level in reservoirs, m	Number of bacteria, 1000/ml, in	
					influent	effluent
Jan. 21	1	2.78	3.64	+0.86	22	29.3
	2	2.75	3.55	+0.80		
Feb. 18	1	4.47	3.54	-0.93	26	38
	2	4.34	3.50	-0.84		
June 18	1	3.34	3.21	-0.13	31	38
	2	3.18	4.09	+0.91		
June 25	1	2.86	3.65	+0.79	38	50
	2	2.76	3.50	+0.74		
July 31	1	3.48	4.23	+0.75	36	44
	2	3.35	4.16	+0.81		
Oct. 15	1	4.80	3.75	-1.05	22	48
	2	4.49	3.49	-1.00		
Nov. 12	1	3.41	3.89	+0.48	12	24
	2	3.45	3.91	+0.46		
Dec. 28	1	4.67	3.94	-0.73	46	64
	2	4.65	3.99	-0.66		

It is important to note that on this occasion we did not discover these "leakages" with the standard method. In past years, however, we have been aware that they were occurring. We explain this by the fact that the standard method for calculating the total bacterial count is less accurate and reveals only very severe disturbances in the functioning of a treatment plant.

In conclusion, grateful acknowledgment is made to Professor A. S. Razumov for his assistance and support in carrying out this work.

SUMMARY

1. Razumov's direct-count method, in the modified form which he suggested, makes it possible to obtain the results of analyses in a short time (15-20 minutes) so that they can be utilized to practical advantage (by preventing release of unsafe water from the supply station or even from individual treatment plants).

2. When the purification process includes chlorination, and preliminary chlorination in particular, it is possible to assess the purification process in its most important aspect—elimination from the water of suspended material consisting of the cells of microorganisms.

3. Wide acceptance of this method for use in water-supply stations should not be precluded by the fact that as yet we have no technique for distinguishing living

cells of microflora from dead cells. At present, it is intended as a sanitary-technical method to supplement but not replace the current sanitary-hygienic methods which are more laborious and time-consuming.

4. The work conducted at our water-supply station has led us to make the following conclusions about the successful operation of purification equipment: a) Up to 75% of the bacterial cells (an average of about 42%) can be retained by a settling basin; b) up to 94% of the bacterial cells (an average of about 47%) can be retained by sand filters; c) cases of the release of unsafe water from purification basins, as well as bacterial "leakages" caused by disruption of the treatment processes, can be detected quickly and easily; d) in storage reservoirs of clear water, multiplication of bacteria can take place in the presence of up to 0.3 mg/liter of residual chlorine. Possible causes for this phenomenon have been noted. Usually the number of bacterial cells drops somewhat in these reservoirs.

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AIR MICROFLORA OF THE CITY OF L'VOV

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The air of populated places does not have characteristic microflora. Bacteria and molds get into the air from soil, water reservoirs, vegetation, and dwellings. Hence, bacterial aeroplankton of populated places reflects local sanitary-topographical and climatic-geographical peculiarities. Consequently, according to Mishustin (1949), Rittenberg (1939), and Zobell (1942), it may even be possible to ascertain, from the presence of certain established species in air microflora, their geographic origin.

However, bacteria and molds or their spores dispersed in air, forming a more or less stable biological system in dust and droplet phases, are subject to the influence of physical factors of the external environment. It is established that low air viscosity constitutes the most important reason for instability of bacterial aerosols. Therefore, microorganisms, particularly those dispersed in large droplets, settle in response to gravitational forces, and sedimentation of bacteria and molds present in droplet nuclei ("wander" dust) occurs much more slowly.

The factor of natural settling of bacterial aerosols from the atmosphere cannot play a significant role, since even a slight activity (0.5-1.5 m per sec), always taking place in external air, supports microorganisms in a suspended state. Bacterial aerosols, naturally established in populated places, are subject to the action of unfavorable conditions in the external environment, among which are variations in temperature, humidity, barometric pressure, precipitation, and ultraviolet radiation.

Although investigation of aerial microflora has been carried out extensively, questions pertaining to the influence of meteorological factors on atmospheric microflora and its qualitative constitution have not been adequately treated in the literature.

Taking into consideration certain meteorological factors, systematic investigation of the degree of bacteriological seeding of air of the city of L'vov, as carried out by us in 1956-1957, offered a possibility to establish a series of characteristics pertaining to the distribution of qualitative composition of microflora under the conditions of defined layout and topography of the underlying surface.

METHODS

Air samples were obtained by suction by means of a modified Krotov apparatus (Pokrovskii, Kishko, Ostranitsa, 1958), which made it possible to carry out studies in the absence of electricity in different regions of the city and in the suburban control location (5 km south of the city).

RESULTS

Air samples (1694 in all) were collected on slides with 2% MPA for studies of the influence of meteorological factors on the qualitative composition of microflora. The presence in air of hemolytic cocci (on Garro's medium and on Turzhetskii's salt and chocolate agar) and of anaerobes (on iron sulfate agar) was established independently.

For this purpose there were carried out, respectively, 2165 and 476 inoculations of air. In almost 90% of the cases, all microorganisms, especially pigmented bacteria and spore-forming aerobes, were Gram-positive. It was established from 4000 microscopic examinations that cocci, particularly micrococci and sarcinae, predominated among the pigment-producing forms (82%). The colorless bacteria consisted primarily of Gram-positive and Gram-negative rods and rarely of cocci. Molds (*Penicillium*, *Aspergillus*, *Cladosporium*) were the predominating fungi (over 60%); yeasts and actinomycetes were present to a lesser extent.

Seasonal averages (for the year 1957) of qualitative composition of air microflora are presented in Table 1 as found in the city's central residential region (location 1, i.e., L1), northern suburbs (L2), railroad square (L3), in the city's natural forest park (L4), and in a country region (L5).

As can be seen from the data in Table 1, the city's air microflora is composed essentially of pigmented, colorless, and spore-forming bacterial species, and, to a lesser degree, of fungi. The pigment formers predominated in the city's air during all seasons, having their relative minimum (32.5-56.5%) in winter, 54.7-60.2% in spring, a relative maximum (40.8-64.6%) in summer,* and 41.2-65.4% in fall. It has been noted that their content was maximal in regions with the most extensive bacterial seeding of air, i.e., in locations 1, 2, and 3. And, conversely, in the park and the suburban locations, there were significantly fewer pigmented microbes.

Similar relationships have been established in studying the distribution of colorless organisms in the city's air.

Thus, airborne colorless bacterial species were also present in maximal quantities in densely populated city districts with extensive street activity.

It is possible to correlate the presence of these airborne groups with the presence of dwellings and human activity. It should be indicated that pigment formation by airborne organisms is more pronounced during the

*As in original—Publisher.

Table 1. Qualitative Characteristic of Air Microflora of the City of L'vov

Location	Average no. of microorganisms per m ³ of air	Qualitative breakdown of microorganisms in %			
		pigmented	colorless	spore-formers	fungi
Winter					
1	708	49.9	17.8	20.6	11.7
2	434	32.5	22.3	25.9	19.3
3	1408	56.5	29.9	4.8	8.8
4	434	41.0	28.7	11.0	19.3
5	339	35.1	15.6	6.2	43.1
Spring					
1	3046	59.8	22.4	15.8	2.0
2	2269	54.7	22.5	14.6	8.2
3	3404	55.5	26.2	14.9	3.4
4	2153	60.2	20.8	11.4	7.6
5	1133	53.8	22.5	11.5	12.2
Summer					
1	6669	64.3	27.3	7.4	1.0
2	1753	60.9	24.7	11.9	2.5
3	5468	64.6	14.6	19.4	1.4
4	2516	40.8	11.4	44.9	2.9
5	1719	48.7	20.8	19.5	11.0
Autumn					
1	6098	56.6	24.3	16.5	2.6
2	1204	44.8	14.9	15.7	24.6
3	3779	65.4	18.6	12.7	3.3
4	1696	41.2	30.6	19.7	8.5
5	952	32.3	5.9	14.7	47.1

clear sunny days of warm seasons. In fact, resistant carotinoids are found intracellularly in these microbial species (Imshenetskii, 1946; Swart-Füchtbauer, 1957), protecting the microorganisms from the lethal action of ultraviolet radiation.

Maximal quantities of spore formers were found in the air over open soil. Thus, fewer organisms were isolated during all seasons at site No. 3 than from the aerial environment of sites 4 and 5. Typically, their content in air reached 44.9% during the summer of 1957 in the vicinity of a developing television center where activity involving an extensive amount of earth was taking place. From this, one can assume that the spore formers, originating in soil, may reflect the nature of the soil surface.

And finally, as can be seen from the table, the number of airborne fungi over a city was relatively high during winter. In the first case, during the winter months, there are few fungi among the molds, and actinomycetes predominate. This, we believe, can be explained by the fact that during 1957, L'vov had an exceptionally mild winter (the average temperature in February was +1.5°), with little snow. In the second place, it is known that fungal spores are quite resistant to low temperatures. However, numerical count of airborne molds during the winter was low: There was up to 40 molds per cubic meter of air. The actual number of airborne city molds increased considerably during spring, summer, and fall, primarily over the extensively planted and growing districts (locations 2 and 4); air in a planted suburban district is seeded more extensively by mold flora in either absolute or relative terms. For example, during the fall these comprised 47.1% of isolations from air microflora.

Consequently, the quantity of airborne molds in general, and of fungi in particular, may reflect the degree of planting in the entire city or in its individual districts.

Hemolytic cocci comprised about 2% of bacterial aeroplankton and were found mostly in air currents of the city's central region, with their numbers reaching up to 100 per cubic meter of air. Morphologically, 69% were micrococci and sarcinae; less frequently, enterococci; and very seldom, real hemolytic staphylococci and greening streptococci (Kishko, 1959).

Anaerobes were less frequently encountered over L'vov (1-2% of positive findings) and were sampled primarily in the air of the central districts.

Studies aimed at establishing the influence of meteorological conditions on air microflora were of interest. It is clear that under natural conditions the interrelationship between meteorological components and air microflora is complex in nature.

Temperature, relative humidity, precipitation, and ultraviolet radiation affect the resistance and viability of microorganisms in the atmosphere in different manners.

The data regarding the degree of bacteriological seeding of air of the centrally located inhabited district, obtained under varying temperature conditions, are presented as a graph in Fig. 1.

As can be seen, in L'vov there is a direct relationship between the air temperature and the degree of its bacterial content: the higher the temperature of air, the more airborne microorganisms. Thus, increasing the temperature from 0 to 10° doubled the content of airborne microorganisms; raising the temperature from 10 to 20° increased the microflora more than 5 times; when the temperature was over 20° the increase was more than sixfold, i.e., the increase in the degree of bacterial seeding of air when the temperature exceeded 20° was less indicative.

The sharp increase in airborne microorganisms with the increase in temperature must be explained not only as due to the influence of temperature, but also as due to the temperature-dependent moments.

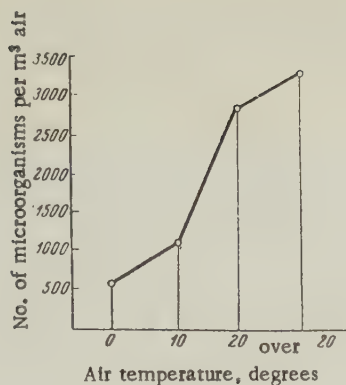


Fig. 1. Influence of temperature on microbial content of air.

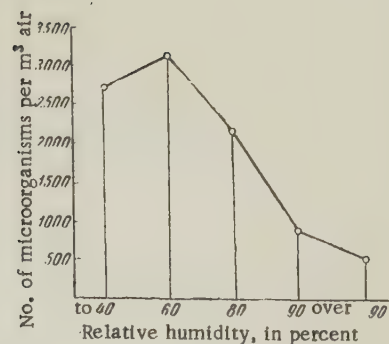


Fig. 2. Influence of relative humidity on microbial content of air.

The soil, the primary source of atmospheric microflora, is covered with snow and is frozen during low temperatures, as a result of which the microorganisms are fixed in the soil. An increase in air temperature is accompanied by drying of the soil and, with the air movements, the earth surface gives rise to microbially filled dust. According to our data there are from 84,000 to 55,000,000 microbes per gram of street dust.

It is known that airborne bacteria are quite sensitive to sharp changes in temperature (Mats, 1945; Omelyanskii, 1936; Rechmenskii, 1951; Brown, 1930; Jacobs, 1939; Skrzynska, 1949). A great number of microorganisms die in air of dehydration due to excessively high temperature. The bactericidal activity of natural ultraviolet rays is intensified in drier air. In subzero temperatures there is a slowing down of microbial multiplication; most of them die in air as a result of cessation or of considerable weakening of physiological processes, especially those involved in metabolic activities of the microbial cell.

However, the airborne microbes possess high adaptability to continuously changing temperature conditions. For example, Proctor (1935) established that one-third of the microbes isolated by him from air at high altitudes developed at 0° and remained viable at -26°.

The stability of bacterial aeroplankton is subject to significant change, depending upon changes in the relative humidity.

As has been shown (Fig. 2), there is a complex relationship between atmospheric relative humidity and the airborne microorganisms.

The curves in Fig. 2 show that a bacterial aerosol is most stable in the middle range of saturation, i.e., 40-60% relative humidity. Increasing the relative humidity to 80% decreases the bacterial content of air by one-third, and if the relative humidity is raised by another 10% (from 80 to 90%), there is a more than threefold decrease in the microbial content of air. And, finally, when the relative humidity exceeds 90%, there occurs a drastic (sixfold) reduction of microorganisms, minimal during precipitation. The decrease in airborne microbes at relative humidities 80-90% and above must be explained by the fact that microorganisms serve as centers of condensation for moisture. Therefore, because of the increase in size and weight of airborne particles, sedimentation of the bacterial aerosol occurs more rapidly.

In drier air, when the relative humidity is lower than 40%, the number of airborne microorganisms is also decreased, and is less than that at 40-60% relative humidity. Apparently, dehydration of microbes plays a significant role. In addition, one must consider that the lower aerial humidity occurs during clear, sunny days. Therefore, decrease in the extent of airborne bacteria is also dependent upon the increased activity of ultraviolet radiation in dry air.

The influence of precipitation upon airborne microorganisms is clear (Fig. 3). It is indicated that there is an inverse dependence between the concentration of airborne microorganisms and the amount of precipitation: the higher the precipitation, the lower the number of airborne microbes, and vice versa.

Two factors are of significance in this consideration. In the first instance, the stability of bacterial aeroplankton is disturbed by direct removal of microorganisms from the air. However, this factor is of greater significance at the beginning of rainfall or snowfall. Thus, should 50% of the microorganisms be "washed out" during the initial 5-10 minutes of a downpour, then subsequently this process slows down considerably.

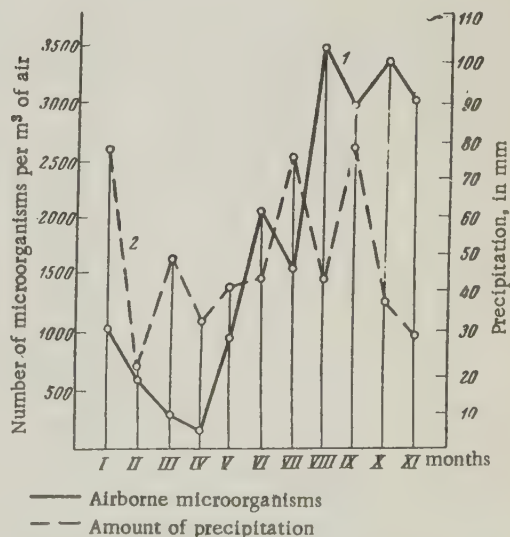


Fig. 3. Influence of precipitation on microbial content of air (1956). 1) Airborne microorganisms; 2) amount of precipitation.

On the other hand, precipitation moistens or covers the ground and holds down the microbes, preventing their entry into the atmosphere during precipitation. However, after precipitation and upon motion of air, the dried ground once again becomes a source of aerial microflora. Because of this, the content of airborne microbes increases with the increase in wind velocity, particularly when this rises above 4.0 m/sec (Fig. 4).

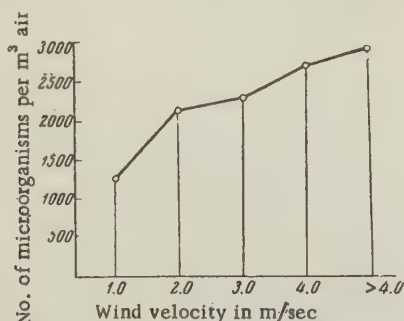


Fig. 4. The effect of wind velocity upon the number of airborne bacteria.

It has been shown that slight variations in barometric pressure have practically no noticeable influence on the count of airborne organisms. Should one take into consideration that increase or decrease in barometric pressure is accompanied by a change in weather, then it becomes understandable that one considers here primarily the secondary influences of barometric pressure on stability of naturally occurring bacterial aerosols. Entirely different relationships were shown by us at higher altitudes. It became apparent that the lowering of barometric pressure with increase in altitude leads to accelerated sedimentation of bacterial aerosol nuclei due to increase in weight due to condensation of cooling moisture on the particles within the nuclei.

And finally, it has been shown that natural ultraviolet radiation in conditions found in L'vov has an adequately expressed bactericidal activity; even at the earth's surface there is an inverse relationship between the concentration of airborne organisms and the intensity of radiation (Kishko, 1960). Thus, during the time of lowest ultraviolet activity ($82 \mu\text{w}/\text{cm}^2$), at 6-8 a.m., there were 2,000 bacteria per m^3 of air, and during the peak of ultraviolet activity (noon to 2 p.m.), there was one-third this amount.

During the cloudy days the action of dispersed ultraviolet rays on aerial microflora was 25-40% less

effective than during the clear, sunny days. The bactericidal effect of natural ultraviolet radiation was decreased three- to fourfold in the fall as compared to the summer.

SUMMARY

1. The predominant forms of airborne microorganisms in L'vov (80%) are the color-forming and colorless species of bacteria.

2. The airborne sporeforming aerobes are indicators of the nature of the soil surface. They are obtained in significant quantities from air over open soil.

3. A maximum number of fungi (particularly molds) has been found in the air over well-planted city districts.

4. An elevation in air temperature promotes drying of the soil, from which surface air motion, particularly that exceeding 4.0 m/sec, lifts a large quantity of bacterium-laden dust.

5. The optimal conditions for stability of natural bacterial aerosols are established when relative humidity is 40-60%.

6. Precipitation significantly purifies the air from microbes (by 50%), but only during the initial stages of the downfall. After that, its role is to moisten the soil, the primary source of air microflora.

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CONDITIONS OF AMYLASE AND PROTEINASE FORMATION IN A SURFACE CULTURE OF *BACILLUS SUBTILIS*

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The *Bacillus subtilis* strain is the richest source of amylase and proteinase and this renders the organism useful for the production of bacterial preparations and enzymes.

Available media which provide accumulations of hydrolytic enzymes for the cultivation of bacteria are decoctions from potatoes, barley, wheat, oats and wheat bran (Imshenetskii, Solntseva, 1944; Imshenetskii, 1944) as well as solid media from wheat bran (Beckord, Kneck, Lewis, 1945; Lulla, Subrahmanyam, 1954).

The present investigation was concerned with the conditions of amylase and proteinase formation during the growth of a surface culture of *B. subtilis* on wheat bran.

METHODS

For the isolation of enzymes we started with three bacterial cultures: *B. subtilis* Japanese, *B. subtilis* Chinese, and *B. mesentericus* of the Institute of Microbiology of the Academy of Sciences, USSR. After comparing the three strains, we selected for our investigation the Japanese strain of *B. subtilis*, as it proved to be the most active in forming amylase and proteinase.

The organisms were surface-cultivated by two methods: on a solid medium from moist wheat bran and as a film on a decoction of the bran.

On a solid medium the organisms were cultivated in Erlenmeyer flasks with a capacity of 250 ml containing 20 g of moist bran which had been sterilized for an hour at 1 atm. The bran decoction for the liquid medium was prepared in the following way: Bran (100 g) was covered with one liter of tap water. After boiling in a water bath for an hour the mixture was squeezed through a piece of linen and poured into flasks. At the end of the period of cultivation, the amylase and proteinase activity and moisture of the culture were determined. The amylolytic capacity (AC) was measured by the Klimovskii and Rodzevich (1949) method of testing for hydrolysis of starch until dextrins unstained by iodine are produced. The proteolytic activity (PA) was determined by measurement (with a nephelometer) of the amount of gelatine left after proteolysis. A detailed description of this method is given in the article by Ivanov (1960).

EXPERIMENTAL

We began our investigation by choosing a suitable medium on which to sow a culture of *B. subtilis*. For this purpose the following agarized media were tested: MPB (meat-peptone broth), MPB + 2% glucose, wort, 50% MPB + 50% brewing wort. As the organisms grew best on the last medium, it was used for all the tests.

In the course of our investigation we also determined the duration of cultivation and storage of the culture which resulted in optimal accumulation of enzymes during subsequent surface growth on bran. We tested cultures of different ages: 24-hr, 48-hr, and 72-hr cultures as well as a 24-hr culture which had been kept before sowing in a refrigerator at +4° for 24 hr, 48 hr and 1½ months, and, finally, a culture which had been transplanted three times at 24-hr intervals. The greatest accumulation of enzymes in the media was produced by 24-hr cultures although the other cultures gave results which were nearly as good. However, in view of these results, in all subsequent investigations we used a 24-hr culture of *B. subtilis* on an agarized medium consisting of equal amounts of MPB and wort.

The organisms were cultivated at 37° as it was found that a higher temperature (30° and 37° were tried) promotes better growth of *B. subtilis* and a greater accumulation of enzymes.

The next series of tests was designed to determine what length of surface cultivation of *B. subtilis* in the form of films in a decoction of wheat bran produced the largest accumulation of amylase. The maximum

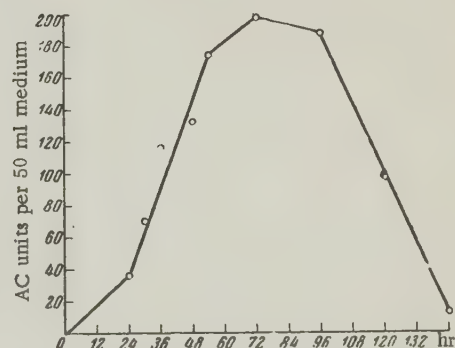


Fig. 1. Effect of length of cultivation of a film culture of *B. subtilis* on the formation of amylase (medium—a 10% decoction of bran).

Formation of Amylase by a Film Culture of *B. subtilis* in Relation to the Thickness of the Layer of the Medium from a Decoction of Bran

Amount of medium in ml in an Erlenmeyer flask with a capacity of 250 ml	AC for the given volume of medium, in units	AC on conversion to 100 ml of medium, in units
30	122	407
50	204	409
70	228	322
90	225	247
110	183	166
130	142	129

amount of amylase was formed after 72 hr of cultivation, when distinct signs of autolysis appeared in the culture (Fig. 1). Moreover, when the conditions of aeration were improved by reducing the thickness of the layer of the medium in the flask, amylase formation considerably increased. The medium was used with particular intensity when only a small amount (30–50 ml) was poured into a 250-ml Erlenmeyer flask to form a layer 10–15 mm thick (Table).

Before investigating the formation of amylase and proteinase on solid media prepared from bran, we compared the different methods of extracting enzymes from surface cultures of *B. subtilis* on bran. Five grams of bran with the culture were thoroughly ground up with quartz sand and left to infuse for an hour at 37° in 50 ml of different solvents: phosphate buffer (pH 5.9 or 7.17) with acetate buffer (pH 5.9) or with water. It was found that enzymes could be best extracted from a phosphate buffer at pH 5.9. For complete extraction one hour was sufficient.

As already mentioned, bran is the best medium for the cultivation of many aerophilic organisms, including *B. subtilis*, as it contains a sufficient amount of nutrient substances (20–26% starch, 1.8–2% nitrogen) (Feniksova, 1956). Even in a moist state it remains loose and has a large surface. However, too much moisture deprives the substrate of porosity and worsens the conditions of aeration, thus making it less suitable for bacterial growth and the accumulation of enzymes. On the other hand, when there is insufficient moisture, the available nutrient substances do not dissolve completely. That is why it was necessary first of all to investigate the effect of moisture of the bran medium on the amount of amylase and proteinase in the *B. subtilis* culture. Figure 2 shows that when bran moisture was within the range 62–75% the maximum amount of amylase was formed (130–170 AC units per 1 g of dry substance). Proteinase was formed more actively when the moisture was greater (70–75%) although even when the bran moisture was 65% the amount of proteinase was only slightly below the maximum level obtained with a moisture of 70–75%. It should be noted, however, that bran containing over 65% of moisture loses its structure and becomes sticky and thus more difficult to use on a large scale. That is why we decided on a moisture of 65%, as it allows for a sufficiently large accumulation of enzymes and makes the medium particularly easy to handle.

We also moistened the bran not with tap water but as recommended in the literature (Lulla, Subrahmanyam,

1954) with $\frac{1}{15}$ M phosphate buffer (pH 7.17) but this had no noticeable effect on the accumulation of enzymes. Moreover, as the salts thus introduced into the medium can complicate the subsequent purification of the enzymes, we did not use a phosphate buffer.

To produce a culture with maximum amylase activity, *B. subtilis* had to be cultivated on bran for 72 hr and for 48 hr for proteinase. However, as 85% of amylase formed within the first 48 hr, we cultivated *B. subtilis* for this period for the accumulation of both amylase and proteinase. Longer periods of cultivation (up to 96 hr) had no appreciable effect on the formation of enzymes, and even after 144 hr the amylase content remained at around 80% of the maximum amount (Fig. 3).

It is noteworthy that the formation of enzymes—especially amylase—by *B. subtilis* is particularly intense once the organisms have ceased to grow and signs of autolysis of the culture begin to appear.

A comparison of the amounts of amylase produced by two different methods of surface cultivation of *B. subtilis* on bran (on a hard medium and in the form of a film on a liquid medium) showed the former method to be the more suitable one. After a 72-hr growth in a film culture there were 409 AC units for every 8.5 g of absolutely dry bran used for the preparation of the medium, i.e., 47 AC units per 1 g of bran; in a hard medium there were three times as many (170 AC units).

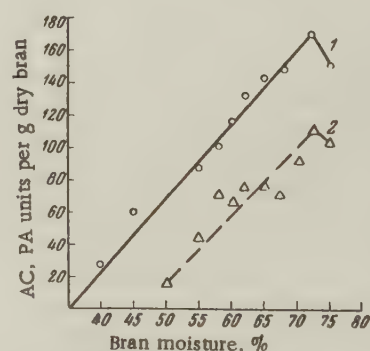


Fig. 2. Effect of Moisture of Medium on the Formation of Amylase and Proteinase in a 48-hr Culture of *B. subtilis* (medium: wheat bran). 1) Amylase (AC units); 2) proteinase (PA units).

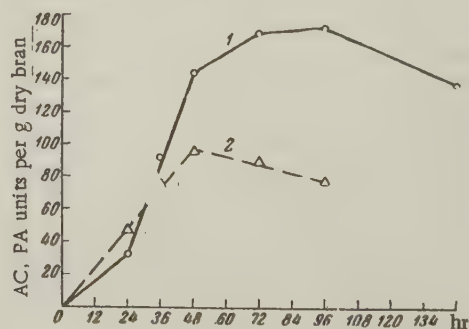


Fig. 3. Effect of Length of Cultivation of *B. subtilis* Culture on Bran on the Formation of Amylase and Proteinase (moisture of medium: 65%). 1) Amylase (AC units); 2) proteinase (PA units).

In order to determine whether this method was suitable for industrial application we carried out several tests in which B. subtilis cultures were cultivated in vessels. The best results were achieved with a 48-hr cultivation and a bran moisture of 65%, which confirms our earlier findings.

From a culture so cultivated we obtained an enzyme preparation with an amylase activity of 1200-1400 AC units per 1 g of dry preparation, or 400 AC units per 1 mg of protein nitrogen. The proteinase activity of these preparations was 350 units per 1 g of dry preparation or 100 units per 1 mg of protein nitrogen. The preparation was produced by extracting the enzymes with water and then precipitating the transparent extract with four volumes of alcohol.

SUMMARY

1. A study was made of the conditions of amylase and proteinase formation in a surface culture of B. subtilis on wheat bran.

2. When cultivated in a 10% extract of wheat bran, a film of B. subtilis produces the greatest amount of amylase after 72 hr of growth in a layer of the medium 10-15 mm thick.

3. In a solid bran medium, 48-hr cultivation of B. subtilis is sufficient for the accumulation of amylase and proteinase. Optimal moisture of the bran is 65%.

4. A greater amount of hydrolytic enzymes is formed by cultivation on a solid moist medium. From such a surface culture a preparation of bacterial enzymes was isolated with an activity of 1200-1400 units amylase and 350 units proteinase per 1 g of dry preparation or 400 units amylase and 100 units proteinase per 1 mg of protein nitrogen.

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WAYS OF INCREASING THE AMYLOLYTIC ACTIVITY OF INDUSTRIAL ENZYME PREPARATIONS PRODUCED BY MOLDS

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In recent years, in the Khar'kov, L'vov, and Rostov enzyme-preparation plants, three strains of mold have been successively introduced into production. These were: *Aspergillus oryzae* No. 81, No. 476-velvety, and No. 476-I. Each successive strain was distinguished from its predecessor by a better capacity to form enzymes, more rapid growth, and greater resistance to infection by harmful microflora. Partly because of this and partly as a result of a better understanding of this new form of production, the amylolytic activity of enzyme preparations produced in the works has increased approximately $1\frac{1}{2}$ times (on an average from 85 to 120-140 SC* units).

Nevertheless, up to the present time the potential capacity of the above-mentioned mold strains to form enzymes has been far from fully exploited in production conditions. As will be seen from Table 1, the average activity of industrial preparations is considerably smaller than that of the control produced by the same mold fungus on the same nutrient media in laboratory conditions. The difference in the indices of activity of laboratory and industrial preparations is as high as 40-50%.

This can only be explained by the fact that in industry all the conditions necessary for the full development of the enzyme-forming capacity of mold have not so far been created.

This matter deserves particular attention, as an increase in the enzyme activity of industrial preparations results in a corresponding increase of the productive capacity of the works. Such an increase in productivity without extra outlay on raw materials, working personnel, etc. would considerably reduce the cost of the product. In order to develop appropriate technological procedures designed to achieve this end, and to eliminate the difference between the activity of laboratory and industrial preparations, it is necessary

first of all to determine the basic causes which bring it about.

The following investigations were concerned with the elucidation of this question.

In the course of our work with strain No. 81 in industrial conditions we found that the initial moisture of the nutrient wheat bran medium had a particularly great influence on the activity of enzyme preparations.

For laboratory conditions, the optimal moisture of a nutrient medium proved to be 60-65%. In industry, because of the different methods of sterilization employed for large amounts of nutrient media, such a high moisture inevitably led to a considerable worsening of its physical structure, i.e., to the formation of lumps and reduction of porosity, which in turn hindered the access of air to the internal layers of the medium and inhibited the growth of the mold, thus creating all the necessary conditions for the infection of the initial culture with bacterial microflora. In industrial conditions a moisture of over 58-59% produced only negative results.

It was also found that in industrial conditions, in spite of aeration of the culture chambers even with air completely saturated with water vapor, cultivation of the mold for more than 24 hr caused a considerable drying of the nutrient medium, so that by the end of the period of cultivation, the moisture of the culture fell to 40 and even 35%. A typical curve representing the change in the moisture content of the nutrient medium during the process of cultivation of the mold in industrial conditions is shown in Fig. 1.

Much of the industrial material is also subject to a fairly regular law: namely, that the activity of the produced enzyme preparation, all other conditions being equal, is determined by the moisture content of the nutrient medium at the last stages of cultivation of the mold. This dependence is clearly illustrated by the inclined straight line in Fig. 2, which is based on the results of tests with strain No. 81. A similar dependence in industrial conditions was found to exist when strains No. 476-velvety and 476-I were used.

In this respect, more favorable conditions obtain in the laboratory, since by cultivating the mold in flasks, the nutrient medium does not dry up but, on the con-

Table 1. Amylolytic Activity of Laboratory and Industrial Enzyme Preparations (Strain No. 476-velvety, cultivated in flasks; initial moisture of medium 60%)

Strain No.	Average yearly amylolytic activity of preparations (SC units)		Activity of industrial preparations as a percentage of the activity of laboratory preparations
	Laboratory	Industrial	
81	150	97	64.7
476-vel.	200	101	50.5
476-I	250	136	54.4

*SC, i.e., saccharifying capacity, is determined by the RTU Ukrainian SSR: 48-58 on enzyme preparations of *Aspergillus oryzae*.

Table 2. Effect of Aeration and Degree of Drying of the Nutrient Medium During the Cultivation of Mold on the Activity of the Resulting Preparation

Test No.	Method of Aeration			Growth of mold	SC of the preparation
	Amount of air per flask (liter/hr)	Relative moisture of air in %	Moisture of culture at end of cultivation in %		
1	Without artificial aeration	—	65.0	Good	185
	10	99-100	64.1		198
	20	99-100	63.7		192
	100	99-100	62.8		186
2	Without artificial aeration	—	65.0	Good	198
	5	92-96	64.3		195
	10	92-96	57.9		173
3	Without artificial aeration	—	64.8	Good	139
	30	85	40.4	Average	72
	60	85	42.0	"	78
	120	85	34.4	Weak	53
4	Without artificial aeration	—	63.0	Good/Average	134
	30	92-95	54.0	Average	98
	60	92-95	48.0	Weak	48
	300	92-95	33.0	No noticeable growth	12

Table 3. Effect of Sterilization Method of the Nutrient Medium on the Enzyme Activity of Preparation

Test No.	Conditions of sterilization of the nutrient medium	Conditions of cultivation of mold	Moisture of nutrient medium in %		SC of the preparation
			Initial	At conclusion of growth	
1	Industrial sterilizer Autoclave Industrial sterilizer Autoclave	In vessels	59.5	38.8	134
		"	59.0	38.1	120
		In flasks	59.5	64.3	200
		"	59.0	63.3	192
2	Industrial sterilizer	In vessels	58.1	36.1	134
		In flasks	58.1	63.2	184
3	Industrial sterilizer Autoclave	In vessels	58.5	46.8	140
		In flasks	58.5	61.3	240
		In vessels	60.0	63.0	248
		In flasks			

with them at the end of the period of growth have a very noticeable effect on the formation of enzymes.

Thus, of all the factors investigated, the initial moisture content of the nutrient medium and its maintenance at that level throughout the whole duration of mold cultivation is the most significant single factor determining the activity of the enzyme preparations.

The results of these tests are presented in Table 3.

On the basis of this conclusion, we created in industry such conditions as would either completely prevent or reduce to a minimum the drying of the nutrient medium. For this purpose, the mold was cultivated not in the usual type of perforated vessels but in vessels with solid bottoms. These vessels, which rested on short supporting legs, were, in their turn, placed in similar but larger vessels which were supplied with a continuous flow of water, the excess of which was removed through decanting tubes. The level in the decanting tubes was so regulated that the running water washed the bottoms of the internal vessels. In this way, the excess heat in the culture was removed by the water through the cooled bottom of the vessel. The experimental vessels were placed on a stand in the industrial culture chamber and protected by special

shields to reduce the multiplicity of changes and the speed of the air flowing over the vessels. For control purposes, ordinary perforated vessels containing an industrial nutrient medium sown with a pure mold culture were placed in the same chamber. The results of these tests are given in Table 4.

These tests demonstrated that when the thickness of a layer of nutrient medium did not exceed 3 cm it was possible to remove most of the physiological heat through the water-cooled bottom of the vessel and to prevent the temperature of the culture from rising beyond 35°. Thus, the evaporation of moisture and the drying of the nutrient medium were reduced to a minimum; the moisture, by the end of the period of cultivation, decreased by not more than 1-2% and, as a result of this, the activity of the preparations considerably increased as compared with the control, and the difference between the laboratory and industrial activity shrank by 7-10%. As it turned out, the temperature of the cooling water proved to be of great importance. When the bottom of the vessel was subjected to excessive cooling, the migrating moisture in the layers of nutrient medium close to the bottom condensed, and as a result these layers became over-

Table 4. Activity of Enzyme Preparations Produced by the Cultivation of Mold in Vessels with Cooled Bottoms

Test No.	Strains	Conditions of Cultivation	Moisture of nutrient medium in %		Maximum temperature	SC of the preparation	Activity of the preparations in vessels with cooled bottoms as a percentage of the activity of preparations	
			Initial	Final			In flasks	In vessels with perforated bottoms
1	476-vel.	Laboratory—in flasks	59.8	65.0	—	185	—	139
		In vessels with cooled bottoms	59.8	58.0	35	167	90	126
		In vessels with perforated bottoms	59.8	50.0	38	133	72	—
2	476-I	Laboratory—in flasks	60.0	64.8	—	189	—	130
		In vessels with cooled bottoms	57.0	55.7	34	175	93	120
		In vessels with perforated bottoms	57.0	48.2	44	146	77	—
3	476-I	Laboratory—in flasks	58.5	64.0	—	240	—	171
		In vessels with cooled bottoms	58.5	59.6	34	224	93	160
		In vessels with perforated bottoms	58.5	40.8	45	140	58	—
4	476-vel.	Laboratory—in flasks	57.8	61.5	—	155	—	161
		In vessels with cooled bottoms	57.8	55.2	35	126	81	131
		In vessels with perforated bottoms	57.8	37.6	40	96	62	—

moist. This, in turn, hindered the aeration of the lower zones of the medium, and the development of the mold was almost completely inhibited and the preparation as a whole displayed very low activity. The temperature of the cooling water must not be lower than 27–28°. The results obtained demonstrate that even in industrial conditions, when there is a large accumulation of mycelium, the enzyme-forming capacity of the industrial strain can be more fully exploited by maintaining the moisture of the nutrient medium at the initial optimal level throughout the whole period of mold cultivation. This shows that it is desirable to replace the currently used industrial method of removing excess heat from the culture chambers by aeration by the cultivation of mold in large fixed vessels with a double bottom cooled by circulating water of a definite temperature.

Long-standing industrial practice had shown that under the existing methods of production, extending the duration of cultivation beyond 24–25 hr did not increase the amylolytic activity of the resultant preparations. But in the light of our findings it is possible that this may have been due to the drying up of the nutrient medium by the twenty-fourth hour with the consequent inhibition of further accumulation of enzymes.

To verify this supposition we carried out tests in industrial conditions using two strains of mold (No. 476-velvety and No. 476-I) which were subjected to prolonged cultivation in vessels with cooled bottoms. The duration of cultivation was extended to 36 hr. Concurrently, the same mold strains were cultivated in ordinary vessels with perforated bottoms, where excess heat was removed by aeration. Beginning with

the twentieth hour, samples were taken every 4 hr to determine the moisture and amylolytic activity of the culture. The results enabled us to follow the course of the amylolytic activity and moisture of the cultures after 24 hr of cultivation.

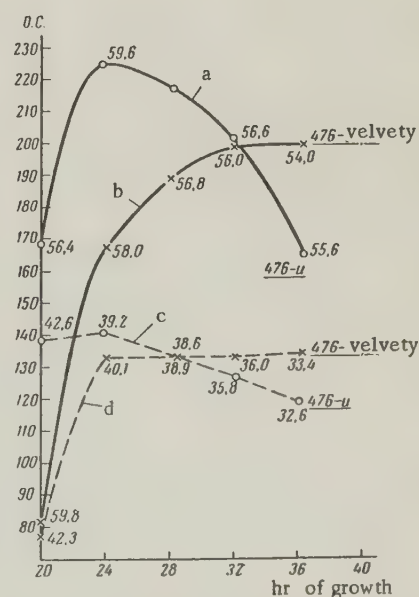


Fig. 3. Course of amylolytic activity and moisture in mold cultures during cultivation in vessels with a cooled bottom and in the usual type of perforated vessels. a, b) SC of preparations from vessels with cooled bottoms; c, d) SC of preparations from vessels with perforated bottoms.

Table 5. Effect of Loosening and Additional Moistening of the Nutrient Medium in the Course of Mold Cultivation on the Amylolytic Activity of Enzyme Preparations (strain No. 81)

Test No.	Moisture of nutrient medium at end of cultivation		Total length of cultivation in hr		SC of preparations		Increase in SC	
	Without loosening	With loosening and moistening	Without loosening	With loosening and moistening	Without loosening	With loosening and moistening	Amylolytic units	%
1	31.8	44.8	24	27	80	146	66	82
2	29.6	45.8	26	29	95	160	65	68
3	33.9	54.2	24	29	96	187	91	95
4	38.6	55.3	24	27	123	196	73	59
5	39.1	50.0	25	30	110	163	53	48

These results are presented in the form of curves in Fig. 3. The numerals at various points on the curves indicate the degree of moisture of the culture. Figure 3 shows that in every case, both when perforated vessels and vessels with cooled bottoms were used, between the twentieth and twenty-fourth hours the two strains displayed a greater or lesser increase in amylolytic activity, whereas in tests in which excess heat was removed by aeration (in vessels with perforated bottoms), during the last 12 hr the amylolytic activity of strain 476-velvety remained at the previous level, and that of strain No. 476-I decreased by 22 units (saccharifying capacity). In vessels with cooled bottoms the differences in behavior of the strains were even more pronounced: Strain No. 476-velvety, after 12 hr additional cultivation, increased the amylolytic activity by 31 units (saccharifying capacity) while the amylolytic activity of strain 476-I, despite the retention of a high degree of moisture in the nutrient medium, decreased from 224 SC units to 163, i.e., by 61 units. Thus, these tests have confirmed our earlier conclusion that when mold is cultivated in conditions under which excess heat is removed by aeration, prolonging the duration of cultivation beyond 24 hr does not increase the amylolytic activity of the preparations. When cultivating mold in vessels with cooled bottoms, the advisability of extending the duration of cultivation depends on the individual properties of the given strain and should be determined experimentally.

In considering the effect of moisture of the nutrient medium on the amylolytic activity of the resultant preparations, a further set of results should be taken into account. Numerous tests carried out in industrial conditions have shown that if, by the fourteenth or fifteenth hour of mold cultivation in vessels with perforated bottoms where excess heat is removed by aeration, the slightly dry culture is subjected to loosening and moistening with sterile water (700-800 ml of boiled water per 1 kg of bran used for the preparation of the nutrient medium), and is then left for a

further period of cultivation in the same vessels, the amylolytic activity of the resultant preparations increases on an average by 60-70% compared with the control. This can be seen in the test results presented in Table 5.

All the experimental results presented in this paper demonstrate the great importance of the moisture of the nutrient medium for the enzyme-forming capacity of the mold. The enzyme activity of industrial preparations can be effectively increased in a practical way by the appropriate mechanization of the processes of loosening and moistening of the culture.

SUMMARY

1. The enzymatic activity of industrial enzyme preparations produced by means of molds is, as a rule, considerably below that of laboratory ones. This difference in the indicators of amylolytic activity of both preparations may be as high as 40-50%.

2. A major factor determining the decrease in the activity of industrial enzyme preparations is the drying of the nutrient medium in the course of cultivation of the mold under industrial conditions. It is practically impossible to exclude this undesirable phenomenon by the procedure used in industry, viz., elimination of excess of physiological heat through ventilation of the culture chambers.

3. It was found that if physiological heat is removed through the water-cooled bottom of the vessel it is possible to prevent the drying of the nutrient medium throughout the period of cultivation of the mold. In this way an appreciable increase in the amylolytic activity of industrial preparations can be achieved, and the discrepancy between the laboratory and industrial activity reduced to 7-10%. A similar result can be obtained by loosening and artificial moistening of the mold culture by sterile tap water during the fourteenth or fifteenth hour of cultivation.

The above procedures provide the possibility of raising the activity of enzyme preparations.

ANTIMICROBIAL PROPERTIES OF SOME ACENAPHTHENE DERIVATIVES

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The search for new antimicrobial substances is a task which is very essential for the national economy and public health.

In examining various organic compounds for antimicrobial properties, we established that acenaphthene, which is a waste product of the coal-tar chemical industry, possesses antimicrobial properties and has a fairly broad antimicrobial spectrum.

Acenaphthene has not yet found application, but it could serve as a cheap source of raw material for the production of antimicrobial substances which are needed for the national economy.

The authors undertook to produce a number of acenaphthene derivatives, so that by employing the method of chemical variations, compounds could be found having stronger antimicrobial properties and better solubility than acenaphthene itself.

A very brief communication has been published on the results of the first stage of our work (Rotmistrov et al., 1957).

The present communication contains material on investigations obtained in 1958. The following acenaphthene derivatives were synthesized and studied.

1. 5-Nitroacenaphthene (Morgun and Leshkina, 1952). Acenaphthene was nitrated with concentrated nitric acid while cooled in ice water and mixed in a solution of acetic acid with a specific gravity of 1.41. From 200 g of acenaphthene, 210 g of 5-nitroacenaphthene was obtained, which constitutes 82% of theoretical yield; the melting point was 101°.

2. 5-Aminoacenaphthene (Hammer, 1930). 5-Nitroacenaphthene was reduced in water-alcohol solution while boiling with sodium hydrosulfite. From 40 g of the nitro product, 27 g of amine was obtained, or 76.7% of the theoretical yield; the melting point was 105°.

3. 5-Hydroxyacenaphthene (Rapoport et al., 1951). 5-Hydroxyacenaphthene was obtained when 5-aminoacenaphthene was heated with dilute sulfuric acid in sealed tubes for 8-10 hours at 200°; the melting point was 124-126°; the yield was 60% of theoretical.

4. 5-Bromoacenaphthene (Graebe, 1902). When acenaphthene was brominated with bromine in chloroform solution, 5-bromoacenaphthene was obtained. The yield was 50% of theoretical; the melting point was 51-52°.

5. 6-Chloro-5-nitroacenaphthene (Dziewonski et al., 1927). Concentrated nitric acid was added with stirring

to a solution of acenaphthene in glacial acetic acid at a temperature of 50°, and then concentrated hydrochloric acid was added at 70-80°. After cooling, crystals of 6-chloro-5-nitroacenaphthene precipitated out; the melting point was 135-138°; the yield was 41% of theoretical.

6. 6-Chloro-5-aminoacenaphthene (Sach et al., 1911) was obtained when 6-chloro-5-nitroacenaphthene was reduced by sodium hydrosulfite in a water-alcohol solution. After repeated crystallization from alcohol, a substance with a melting point of 138-140° was obtained; the yield was 20% of theoretical.

7. 5-Hydroxy-6-chloroacenaphthene (Dziewonski et al., 1927). When 6-chloro-5-aminoacenaphthene was diazotized with sodium nitrate in dilute sulfuric acid and then steam-distilled, a slight yield of 5-hydroxy-6-chloroacenaphthene was obtained. The melting point was 120-122°.

8. 3-Sulfoacenaphthene (Dziewonski et al., 1924). 3-Sulfoacenaphthene was obtained by nitration of acenaphthene with chlorosulfonic acid at 0° in a solution of nitrobenzene. When it was salted out with sodium chloride, the Na salt of 3-sulfoacenaphthene was obtained. 3-Sulfoacenaphthene was obtained by the action of hydrochloric acid on a concentrated aqueous solution of this salt. The melting point was 120°.

9. 2-Sulfonaphthalic acid (Dziewonski et al., 1924). 2-Sulfonaphthalic acid was obtained by oxidizing the sodium salt of 3-sulfoacenaphthene with sodium bichromate while boiling in acetate solution. The yield of the sodium salt of 2-sulfonaphthalic acid was 62% of theoretical. The free acid was separated out with hydrochloric acid. The melting point was 165-166° (decomposition).

10. 2-Hydroxynaphthalic acid (Dziewonski et al., 1927). Alkaline fusion of the sodium salt of 2-sulfonaphthalic acid with KOH at 180° gave the sodium salt of 2-hydroxynaphthalic acid. When the latter was acted upon by hydrochloric acid, 2-hydroxynaphthalic acid was obtained in a yield of 96% of theoretical. The melting point was 340° (decomposition).

11. Naphthalic acid (Graebe and Gfeller, 1892). When acenaphthene was heated in acetate solution with sodium bichromate, naphthalic acid was obtained. The melting point was 270°.

12. Naphthalimide (Graubert, 1895) was synthesized by heating a mixture of naphthalic anhydride with ammonia at 60–70°; the yield was nearly quantitative; the melting point was 300°.

13. The imide of 4-nitronaphthalic acid (Okazaki et al., 1957) was obtained similarly to naphthalimide.

14. The N-phenylimide of 5-nitronaphthalic acid (Okazaki et al., 1957). The N-phenylimide of 5-nitronaphthalic acid was obtained when 5-nitronaphthalic acid anhydride was boiled with aniline for four hours, followed by cooling and washing with acetic acid. The melting point was 280°; the yield was 50% of theoretical.

15. 4-Bromonaphthalic anhydride (Rull and Thompson, 1937) was synthesized by brominating naphthalic anhydride in alkaline medium.

When the compound obtained was nitrated with nitric acid, the following was obtained:

16. 4-Bromo-3-nitronaphthalic anhydride (Rull and Thompson, 1937). The yield of the compound obtained was insignificant.

17. Naphthalic anhydride (Kiprianov and Dashevskii, 1934). Synthesized by oxidizing acenaphthene in acetic acid. Yield 60%; melting point 265°.

Acenaphthene and its derivatives are practically insoluble in water, with the exception of 3-sulfoacenaphthene, 2-sulfonaphthalic acid, and naphthalic acid. Acetone proved to be the most suitable solvent for studying the antimicrobial properties of the acenaphthenes; 0.5–1% solutions of the compounds were obtained in this solvent. The solubility in ethanol was considerably lower. The water-soluble derivatives of acenaphthene are poorly soluble in ethanol and acetone.

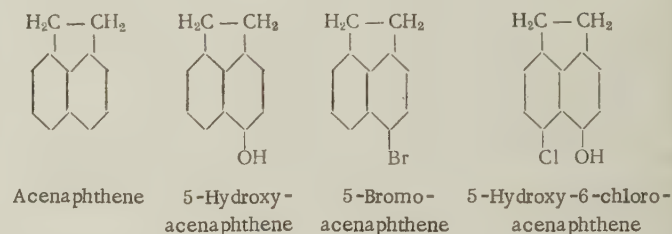
The antimicrobial properties of acenaphthene and its derivatives were studied by the serial dilution and titration series methods. When bacteria served as the test material, titration series were set up in MPB. When testing the effect on mycelial fungi, titration series were set up on Sabouraud's agar medium, while for yeast-like fungi, they were set up on dilute, 7° Balling beer wort. Either 0.5% alcoholic or acetone solutions served as the initial solutions of acenaphthenes, while for substances soluble in water, 1% aqueous solutions were used.

Table 1. Antimicrobial Properties of Acenaphthene and Its Derivatives

Compounds	Inhibition of microorganisms in dilutions $\times 10^3$			
	S. aureus No. 209	E. coli	Tricophyt. gypseum	Candida albicans
Acenaphthene	10	10	10	10
5-Aminoacenaphthene	20	30	10	10
5-Hydroxyacenaphthene	200	200	20	40
5-Bromoacenaphthene	100	10	10	10
6-Chloro-5-nitroacenaphthene	1	1	1	1
6-Chloro-5-aminoacenaphthene	20	20	2	2
5-Hydroxy-6-chloroacenaphthene	200	20	20	20
3-Sulfoacenaphthene	10	—	10	—
Naphthalimide	20	30	—	10
4-Bromonaphthalic anhydride	20	10	—	—
4-Bromo-3-nitronaphthalic anhydride	200	20	—	—

Data on the bacteriostatic and fungistatic activity of acenaphthene and its most active derivatives are given in Table 1.

As seen from Table 1, the introduction of halogens and of the hydroxyl group into the acenaphthene ring increased the antimicrobial activity of the compounds. Thus, whereas acenaphthene itself inhibited the growth of four of the microorganisms which we tested in a dilution of 1:10,000, 5-hydroxy-acenaphthene, 5-bromoacenaphthene, and 5-hydroxy-6-chloroacenaphthene inhibited the growth of *Staphylococcus aureus* in dilutions of from 1:100,000 to 1:200,000. The bacteriostatic effect of these compounds with respect to the representative of the Gram-negative group of bacteria — *Escherichia coli* — was apparent in dilutions of from 1:1000 to 1:20,000. The fungistatic activity of these acenaphthene derivatives coincided with the bacteriostatic activity with respect to *E. coli*. The structural formulas of the acenaphthenes which showed the highest antimicrobial activity are given:



The oxidation of acenaphthene (breaking of the acenaphthene bridge) decreased the antimicrobial activity of the compounds, and only 4-bromo-3-nitronaphthalic anhydride approached the most active compounds mentioned in strength of antimicrobial effect.

It should be noted that naphthalimide as well as 5-aminoacenaphthene inhibited Gram-negative bacteria more strongly than it did Gram-positive ones. Thus, while they were effective against *E. coli* at a dilution of 1:30,000, they inhibited the growth of *S. aureus* 209 only at a dilution of 1:20,000.

The bactericidal effect of acenaphthene and some of its derivatives showing considerable bacteriostatic activity was also studied. The data of these experiments are given in Table 2.

Table 2. Bacteriostatic and Bactericidal Effect of Some Acenaphthene Derivatives

Compounds	Effect on S. aureus 209 in dilutions $\times 10^3$	
	bacteriostatic	bactericidal
Acenaphthene	10	10
5-Hydroxyacenaphthene	200	20
5-Bromoacenaphthene	100	10
5-Hydroxy-6-chloroacenaphthene	200	1
4-Bromo-3-nitronaphthalic anhydride	200	20

As seen from Table 2, acenaphthene and its derivatives have a somewhat lower bactericidal than bacteriostatic activity.

In connection with the fact that in practice, disinfectants must inhibit mixtures of microbial populations

belonging to different species, as well as microbial associations encountered in various secretions, experiments were conducted to study the effect of three acenaphthene derivatives on associations of Staphylococcus aureus with E. coli, Proteus, and B. pyocyanea. The data are given in Table 3.

Table 3. The Effect of Some Acenaphthene Derivatives on Staphylococcus aureus in Pure Culture and in Associations

Compounds	Bacteriostatic effect in dilutions $\times 10^3$ on:			
	microbial associations			control
	<u>S. aureus</u> <u>E. coli</u>	<u>S. aureus</u> <u>Prot. vul-</u> <u>garis</u>	<u>S. aureus</u> <u>B. pyocyanea</u>	<u>S. aureus</u>
Naphthalimide	10	10	10	20
4-Bromonaphthalic anhydride	10	10	8	20
5-Amino-6-chloro-acenaphthene	10	10	10	20

It is seen from Table 3 that the antimicrobial properties of the acenaphthenes decreased by about one-half when acting on associations of staphylococcus with Gram-negative bacteria.

Preliminary experiments were conducted to study the adaptation of S. aureus, P. vulgaris, E. coli, and Bact. pyocyanea to some acenaphthene derivatives. As a result of 10-12 passages of the cultures on media containing successively increasing amounts of acenaphthenes, the resistance of the test organisms increased five- to tenfold.

Thus, among the 18 acenaphthene derivatives which we obtained by means of organic synthesis, there are compounds which have considerably greater antimicrobial properties than phenol and certain other widely used antiseptics. The fact that 5-aminoacenaphthene

and naphthalimide have stronger bacteriostatic effects on Gram-negative bacteria than on Gram-positive ones deserves particular attention.

At present, it would be premature to predict the area of application of the acenaphthenes. There is reason to believe that certain of the acenaphthene derivatives characterized by simplicity of synthesis, adequate solubility, and high antimicrobial activity will find application as new, inexpensive antiseptics and disinfectants.

SUMMARY

1. Acenaphthene, a waste product of the coal-tar chemical industry, has considerable antimicrobial properties; in biological titrations, it inhibits Staphylococcus aureus, E. coli, Trichophyton gypseum and the Candidae at a dilution of 1:10,000.

2. Acenaphthene is insoluble in water and poorly soluble in the more available organic solvents, which is an obstacle to its utilization as a new disinfectant.

3. Taking into consideration that acenaphthene has not yet found application and is an extremely cheap raw material, it seemed expedient to study a number of its derivatives for the purpose of finding new antimicrobial compounds.

4. Among the compounds which were studied, 5-hydroxyacenaphthene, 5-hydroxy-6-chloroacenaphthene, 5-bromoacenaphthene, 5-aminoacenaphthene, 6-chloro-5-aminoacenaphthene, naphthalimide, and 4-bromo-3-nitronaphthalic anhydride were distinguished by the most pronounced bacteriostatic and fungistatic properties.

5. Staphylococcus aureus in associations with Gram-negative bacteria was twice as resistant to the effect of some acenaphthene derivatives as a pure culture of staphylococcus.

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GAMMA-RADIATION AS A DISINFECTING AGENT FOR BOOKS INFECTED WITH MOLD SPORES

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At the present time, extensive studies are being carried out in the USSR and abroad in the utilization of γ -radiation for the sterilization of foods and different substances (Brownell et al., 1956; Bondarev, 1958; Meisel', 1955; Proctor et al., 1956; Khennan, 1957; Chernyaev, 1957).

According to Proctor and Goldblith (1956), cold sterilization by means of γ -radiation is being already used for medicinal substances—antibiotics, vitamins, and so forth. Investigations of the nature of biological effects of γ -radiation established that doses of the order of several hundred thousand rep are lethal for microorganisms. Or, according to Meisel' (1955), doses of the order of several tens of thousands of roentgens inactivate 50% of yeast cells, and doses of the order of several millions of roentgens must be used to destroy microbial cells. Brownell and Balmer (1956) established that complete sterilization of packing material infected with *Escherichia coli* and *Bacillus stearothermophilus* occurs after exposure to 2,000,000 fermis.

Proctor and Goldblith (1956) demonstrated that the magnitude of the sterilizing dose depends upon the characteristics of the microorganisms in question. This fact has been confirmed by a series of other investigations. Thus, Bridges, Olivo, and Chandler (1956) established, in studying the relative stability of microorganisms, that certain species of yeasts, capable of withstanding irradiation at 1,800,000 fermis, are the more resistant of the fungi. According to Pepper, similar resistance to γ -radiation was shown by spores of certain bacteria (Bridges et al., 1956). Fungi, *Penicillium*, and *Aspergillus* are less resistant to irradiation. The lethal dose for certain species of *Aspergillus* equals approximately 300,000 fermis, and for *Penicillium* it is 200,000 fermis.

At present, no data are available regarding the effects of γ -radiation on the mechanical properties of the sterilized materials. It is known that cellulose and similar materials show a decrease in tensile strength after exposure to high doses of radiation. Seaman showed that the dose of radiation which is demaging to cellulose is considerably higher than the sterilizing dose (from Brownell et al., 1956).

At present, the only method of disinfecting books in libraries is by means of formalin. Books placed in a chamber are subjected to the action of its fumes.

Formalin is an effective agent, and there is hardly any change in the mechanical strength of paper subjected to its action. However, in disinfecting books in such a chamber only the surfaces accessible to formalin are rendered free of infection, and places closed to formalin (backs, stuck pages) remain contaminated. Successful disinfection of books by formalin does not take place even in an evacuated chamber. Therefore, libraries are in need of more effective methods of disinfection.

The purpose of this study was to determine the possibility of utilization of γ -radiation for disinfection of books. In this connection, a study was made of the relative resistance of different species of book-damaging fungi to γ -radiation and of changes in mechanical properties of paper treated by γ -radiation.

The radioactive isotope Co^{60} was used as a source of γ -radiation. Mold spores grown on books or deposited on samples of paper, as well as samples of different types of papers, were exposed to radiation. All samples were placed inside books. The books were packed in groups in a cardboard box and were irradiated in the inner zone of a cylindrical cobalt γ -emitter.

The effect of irradiation on fungi was established by results of spore development at 27°. If mold spores did not germinate within 10 days after inoculation on nutrient medium (wort agar), then it was considered that mold spores lost their viability as a result of irradiation; if a mold germinated, then γ -radiation was not lethal to spores.

Filter papers (D-1, 4 cm) infected with spore suspension of a pure ten-day-old mold culture were used in studying the resistance of fungal spores to γ -radiation.

A mixture of sand and spores in water was prepared in advance to yield a spore suspension; after mixing, the mold suspension was separated from sand by decanting, and the filter papers were immersed individually. After drying, the samples were placed in sterile paper envelopes which were placed in a book. The experiments were repeated five times.

After irradiation, each sample was transferred into 10 ml of wort agar medium (melted and cooled to 40°) which was poured into a Petri dish after careful agitation by means of a glass rod.

The effectiveness of action of γ -radiation on spores of artificially book-grown fungi was established by

Relative Resistance of Fungi to Gamma Radiation

Fungus	Max. radiation dose allowing devel. of fungi, all replicates (10 ³ fermi)	Min. radiation dose inhibiting devel. of fungi, all replicates (10 ³ fermi)
Asp. ruber	—	—
Oospora crustacea	—	—
Pen. herquei	—	—
" simplicissimum	—	—
Rhinotrichum parietinum	—	—
Chaetomium elatum	—	150
Rhinotrichum bloxami	—	150
Sporodesmium echinulatum	—	200
Trichoderma lignorum	100	200
Asp. repens	150	200
Pen. fellutanum	150	200
" roqueforti	150	200
" variabile	150	250
Asp. versicolor	150	250
Chaetomium globosum	150	250
Paecilomyces varioti	150	250
Pen. viridicatum	150	250
" cyaneo-fulvum	200	250
" oxalicum	200	250
" steckii	200	250
" palitans	150	300
" tardum	250	300
" notatum	200	350
" citrinum	250	350
Sporodiniopsis dichotomus	250	350
Mucor racemosus	300	350
Pen. chrysogenum	300	350
Stachybotrys atra	300	400
Stemphylium piriforme	400	550
Stemphylium botryosum var. botrytis	500	650

means of inoculation of mold growth from the irradiated book on wort agar medium; at least ten samples were taken from different parts of a book.

The results of experiments dealing with relative stability of molds are summarized in the table. From the data it is seen that spores of the molds *Stemphylium* and *Stachybotrys atra* are the most resistant. The least resistance was shown by *A. ruber*, *O. crustacea*, *P. herquei*, *P. simplicissimum*, and *Rhinotrichum parietinum*, which were inhibited after exposure to 100,000 fermis (the lowest dose used in the experiment). Species of *Penicillium* vary in their stability (to radiation).

It has also been established that strains weakly stable to γ -radiation were also most sensitive to heat.

Experiments dealing with the action of γ -radiation on fungus-infected books (artificially infected with *P. chrysogenum*) substantiated the data pertaining to relative stability. Mold growth was observed on artificial nutrient medium in test-tubes after exposure to 250,000 fermis. The action of the next higher dose used in the experiment, 500,000 fermis, and of higher doses did not allow the development of *P. chrysogenum*. Thus, the lowest dose of radiation destructive to mold development was between 250,000 fermis and 500,000 fermis. The study of relative stability of molds showed that 350,000 fermis is the lowest lethal dose for spores of *P. chrysogenum*.

The data relative to conditions guaranteeing a more complete disinfection of books are of basic practical interest. Experimental results showed that only the

action of 650,000 fermis completely prevents development of resistant molds harmful to books.

In order to determine the influence of time on the comparative change in the developmental ability of irradiated and unirradiated spores, we counted the number of germinating spores after four months' preservation subsequent to irradiation of experimental samples. During this time, the irradiated and the control samples were kept together under laboratory conditions. The results showed that the number of germinating irradiated spores is significantly reduced as compared to that of control spores. For example, the number of developing control spores of *Stemphylium botryosum* var. *botrytis* decreased by 57%; of those irradiated with 250,000 fermis, by 68%; and of those subjected to 500,000 fermis, by 93%. Therefore, even if mold spores are not killed rapidly after irradiation, they are so affected by γ -radiation that they lose their ability to germinate more rapidly than do the unirradiated spores.

Observations of mold growth under experimental conditions on irradiated (1,100,000 fermis) paper compared to that on unirradiated paper showed that all molds develop similarly on control (unirradiated) and on irradiated paper. Therefore, prior irradiation of paper is without significance for subsequent development of molds on it.

Irradiated paper does not undergo any outwardly apparent changes. Testing of physicomechanical properties showed that certain changes do take place in the paper. Changes in resistance to tearing and stretching are not apparent even after irradiation with a dose of 4,000,000 fermis. Resistance to breaking (number of double creases) is altered even after exposure to a disinfecting dose of 700,000 fermis. Resistance to breakage increases in art paper and decreases in newspaper stock and in # 1 printing paper; with an increase in radiation dose, there occurs a more significant decrease in susceptibility to breakage.

SUMMARY

1. Results of the present study have confirmed the findings of Bridges, Olivo, and Chandler that fungal species vary in their resistance to γ -radiation.

2. Among the molds frequently damaging books, the most resistant to γ -radiation proved to be the *Stemphylium* genus and *Stachybotrys alba*; the most sensitive were *Aspergillus ruber*, *Oospora crustacea*, *Penicillium herquei*, *P. simplicissimum*, and *Rhinotrichum parietinum*.

3. With a minimal dose of 650,000 fermis, it was possible to achieve complete disinfection of books from spores of molds.

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THE FORMATION OF A CRIMSON PIGMENT IN ACTINOMYCES RIMOSUS

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In the course of investigating the morphocultural characteristics of *Actinomyces rimosus* strain 101, our attention was drawn to the following phenomenon:

During cultivation of *A. rimosus* (the source of oxytetracycline) in petri dishes on potato or corn agar, when bacteria entering from the air developed an adjacent colony, as a rule there appeared a bright crimson staining of the medium. Not every microorganism entering from the air thereby brought about formation of the crimson pigment, but only certain species of bacteria.

Appearance of the crimson pigment was so specific that it could serve as an indicator of contamination of the *A. rimosus* culture with extraneous microflora.

We have not encountered in the literature a description of similar phenomena with reference to *A. rimosus*.

In view of this, it was to our interest to find out which bacteria when grown in a mixed culture with *A. rimosus* would be contributory in bringing about formation of the crimson pigment; on which culture media the crimson pigment would form; whether all the *A. rimosus* cultures at our disposal would form the crimson pigment in the presence of bacteria; whether other species of the Actinomycetaceae would form the crimson stain; and whether appearance of the crimson pigment was related to a change in the pH of the medium and to antibiotic activity.

The experiments were carried out in replicate; the incubation temperature was 27-28°. Antibacterial activity was determined by the agar diffusion method; the pH of the culture liquid was determined by a potentiometer. The constituents and preparation of the culture media were as follows (%):

1. Potato medium. 200 g of thoroughly scrubbed potato was grated through a fine strainer. The grated pulp was then rinsed three times and filtered through a double layer of muslin. The mash was discarded and the rinsings were set aside for the starch to precipitate, after which a liter of water was decanted and to this were added $(\text{NH}_4)_2\text{SO}_4$ -0.35; NaCl-0.3; calcium carbonate-0.2; glucose-1.0; the precipitated starch was then stirred into this mixture.

2. Corn medium. Corn extract-1.0 (dry measure); $(\text{NH}_4)_2\text{SO}_4$ -0.2; NaCl-0.05; glucose-0.5; calcium carbonate-0.2; starch-1.0.

3. Pea medium. Pea extract-10.0; peptone-0.5; NaCl-0.5; glucose-1.0.

4. Modified Czapek medium (Medium No. 1). MgSO_4 -0.05; K_2HPO_4 -0.05; NaCl-0.05; KNO_3 -0.1; glucose-

1.0 All these media were solidified by the addition of 2% agar.

Deep cultivation was carried out in: 1) corn medium—corn extract-0.5; $(\text{NH}_4)_2\text{SO}_4$ -0.4; NaCl-0.5; calcium carbonate-0.5; starch-2.5; 2) synthetic medium— $(\text{NH}_4)_2\text{SO}_4$ -0.5; NaCl-0.5; corn extract ash-0.03; calcium carbonate-0.05 (sterilized separately); starch-3.0; 3) potato medium, the composition of which is given above.

To clear up the question of which bacterial flora are contributory to the formation of crimson pigment in the source of oxytetracycline we prepared streak plates of *A. rimosus* with bacteria of a known species on potato agar. The species used were: *Bacillus subtilis*, *Bacillus mycoides*, *Staphylococcus aureus*, *Escherichia coli*, *Sarcina lutea*, and a form we had isolated which is closely related to the Micrococci.

The pigment appeared only on those plates containing mixed cultures of *A. rimosus* with *B. subtilis*, *B. mycoides*, or *E. coli*; after two days the medium, and sometimes the vegetative mycelium of the actinomycete, was stained a bright crimson color.

Subsequent investigations were conducted only with *B. subtilis*.

To elucidate the role of nutrient medium in formation of the crimson pigment we used the following solid media: potato, corn, pea, meat-peptone, and No. 1. Seeding was carried out as on the first occasion. The most intensive pigment was formed, as a rule, with cultivation on potato agar; it was somewhat fainter on corn; a faint pigment appeared on the pea agar, while it did not occur at all on meat-peptone agar (MPA) or medium No. 1.

In testing the capacity of various strains of *A. rimosus* to form the crimson pigment in the presence of *B. subtilis*, we found that they did not all have this property.

Of the 24 *A. rimosus* cultures investigated [four initial strains and 20 variants obtained experimentally (Rudaya, 1959)] in five variants no crimson pigment was formed when cultivated in association with *B. subtilis*.

We also observed no formation of crimson pigment under these conditions when testing the sources of such antibiotics as streptomycin, streptothricin, chloramphenicol, chlortetracycline, and unidentified actinomycetes of the yellow, red, brown, gray, and colorless groups.

Table 1. Effect of *B. subtilis* on Formation of the Crimson Pigment, the pH, and the Antibiotic Activity of the *A. rimosus* Culture Liquid

Actinomycete culture	Test conditions	Formation of crimson pigment	pH	Activity, given in units/ml
101	Corn medium—control	—	6.9	500
	" " + <i>Bac. subtilis</i>	+	6.9	515
	Potato medium—control	—	7.1	Not determined
	" " + <i>Bac. subtilis</i>	+	7.0	"
9-1-R ₂	Corn medium—control	—	7.0	310
	" " + <i>Bac. subtilis</i>	+	6.8	300
	Potato medium—control	—	7.0	Not determined
	" " + <i>Bac. subtilis</i>	+	7.0	"

In order to investigate the connection between formation of the crimson pigment, change in the pH of the medium, and the antibiotic activity of *A. rimosus*, submerged cultivation was carried out (shaking flask technique) in corn and potato media. We inoculated the media with agar cultures of *A. rimosus* strain 101, and its variant, 9-1-R₂, and added 0.2 ml of *B. subtilis* suspension, containing 2,000,000,000 spores per ml. The bacterial culture was not added to the control flasks. After three days we examined the flasks and measured the pH and the antibiotic activity. These data are presented in Table 1.

The results presented in Table 1 show that the addition of the bacterial culture to *A. rimosus* brought about formation of the crimson pigment; this did not produce changes either in the pH of the culture liquid or in its antibiotic activity.

In other tests we inoculated *B. subtilis* spores into one-, two-, three-, and four-day subsurface cultures of *A. rimosus* (strain 101), but the results of these tests were discrepant: Crimson pigment occurred in some cases and not in others. According to our observations, formation of the crimson pigment was better with surface cultivation.

It is essential to note that we encountered the formation of pigment of this nature under other conditions as well. When *A. rimosus* strains 101, A, 9-1, and II were cultivated at 37°, 41°, and 45°, appearance of the crimson pigment in the medium was noted on the third to fifth days of growth. Cultivation was in test tubes on solid potato, corn, and No. 1 media. Growth of all strains on all media was very good at 37° and 41°, but the crimson pigment was denser on potato agar than on corn, while it did not appear at all on No. 1 medium. The cultures at 44-45° grew much more poorly and the crimson pigment was also much fainter at this temperature. The deepest pigment was observed at 40-41°. No formation of crimson pigment took place in these strains when cultivated at the usual temperature (26-28°) on the media indicated. In one of the *A. rimosus* variants obtained experimentally (9-1-R₂), however, a characteristic was its secretion of a pigment of this color at a temperature of 26-28° and only on potato agar.

It should be mentioned that with subsurface cultivation of some *A. rimosus* cultures (9-1, II, and 9-1-R₂) on synthetic medium crimson pigment occurred (mycelia and liquid were stained) with varying intensity,

Table 2. R_f Values of the Pigment in Ascending Paper Chromatography

System	R _f in mm
Methanol	0.55
Butanol, saturated with water	Diffuse spot; 0-0.13
Butanol-pyridine-water (1:0.6:1)	0.23
Butanol-acetic acid-water (2:1:1)	0.60
100% acetone	0
50% aqueous acetone	0.66
Water, saturated with butanol	0.86
Distilled water	0.78
3% NH ₄ Cl	0.76
Benzene-acetic acid-water (2:2:1)	0.61

whereas on corn media in these conditions the pigment did not appear at all. In all the above cases the *A. rimosus* was a pure culture and successive transfers to sterile plates revealed no contamination with extraneous microflora.

Several properties of the pigment were investigated.*

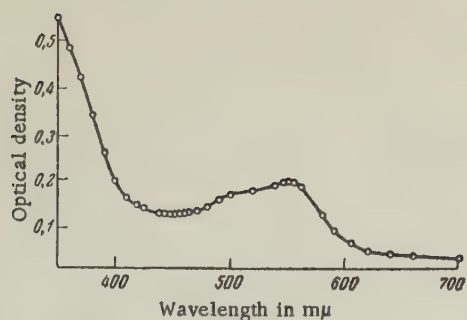
The culture liquid was saturated with ammonium sulfate and was extracted with acetone or isopropanol. The organic phase, which was yellow in color, was discarded. The aqueous phase, crimson in color, was evaporated in a vacuum until thoroughly desiccated and the residue was extracted with methanol.

The pigment did not extract from the culture liquid with butanol, diethylene and petroleum ethers, chloroform, carbon tetrachloride, or ethyl acetate. The pigment did not dissolve in carbon tetrachloride and diethylene ether; it was moderately soluble in dimethylformamide, while it did dissolve in water and methanol.

The pigment had a limited ability to inhibit *Staph. aureus*. It did not fluoresce in UV light. In acid and alkaline reactions the pigment did not change color. On this basis it can be differentiated from pigments of the anthocyanin group and also from the pigmented antibiotics which have indicator properties (litmocidin, rhodomycin, actinorhodine, coelicolorin, violarin, and others).

In ascending filter paper chromatography on a filter paper with rapid capillary action in the water-saturated solvents petroleum ether, chloroform, carbon tetrachloride, ethyl acetate, and amyl acetate, the pigment did not migrate from its original site on the paper. The R_f values in other solvent systems are presented in Table 2.

*We wish to acknowledge the helpful assistance of N. O. Blinov in studying the properties of the pigment.



Absorption spectrum of the pigment in visible light (in methanol solution).

Maximum absorption of the pigment in methanol in visible light (Fig.) was in the green zone at a wavelength of 550 mμ.

The cause for the appearance of the pigment under a variety of conditions (presence of bacterial flora, growth on several solid media at slightly raised tem-

peratures, appearance of the crimson pigment in the indicated strains of *A. rimosus* during submerged growth on synthetic agar medium) remains obscure.

SUMMARY

During growth of *A. rimosus* in a mixed culture with certain bacteria (*B. subtilis*, *B. mycoides*, *E. coli*, and others) on potato and corn agar the formation of a crimson pigment was observed. This same pigment also occurred during cultivation of *A. rimosus* on the above media without bacteria: 1) at 40–41°; 2) in submerged growth of certain strains of *A. rimosus* on synthetic medium; 3) in one of the variants of *A. rimosus* on potato medium at 26–28°.

Several properties of the pigment were investigated.

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SPECIES IDENTIFICATION OF THE SCHIZOSACCHAROMYCES

GENUS OF YEASTS

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The unicellular yeastlike fungi, lacking mycelia and reproducing by fission, are grouped together in the family Schizosaccharomycetaceae, which includes two genera: *Schizosaccharomyces* and *Octosporomyces*. The genus *Schizosaccharomyces* is represented by two species: *S. pombe*, isolated and studied by Linder in 1893, and *S. acidodevoratus*, which Chalenko studied in 1941 (Kudryavtsev, 1954). The *S. acidodevoratus* yeasts decompose malic acid during the fermentation of fruit wines and occur as destructive agents in the wine-making industry.

In species identification of the fission yeasts *S. pombe* and *S. acidodevoratus*, the effect of the yeasts on malic acid can be established by inoculating them into fresh apple juice, which is rich in malic acid; it is then possible to determine the species according to breakdown of the malic acid during the fermentation process (Chalenko, 1941; Kudryavtsev, 1954; Gnutenko et al., 1957).

During the investigation of schizosaccharomycetes which had been fermenting woodwort at a series of industrial hydrolysis plants a need arose for species identification of the yeasts isolated.

In the tests we used commercial apple juice and crystalline malic acid.

Crystalline malic acid was added at concentrations of 0.8%, 1%, 3%, and 6% to synthetic medium composed of: glucose—2.5%, phosphate bond—1.0% (1/15 M solution KH_2PO_4 ; pH—5.59), and yeast autolyzate—2.0%; the malic acid was also added to woodwort at concentrations of 0.8% and 6%. The concentration of yeasts in the experiment was 20 g per liter. The sugars fermented were determined by a boiling-point curve and acidity was determined by titration with 0.1 N NaOH.

Method for Volumetric Acid Analysis

One ml of the fluid under study was diluted into 25 ml of water and this was then boiled and titrated while

hot with 0.1 N NaOH in the presence of phenolphthalein. Acidity was expressed in ml of 0.1 N NaOH used per ml of medium.

Constituents of the media employed in the experiments are presented in the table.

The species to be identified were stock cultures from the Kos'va (*S. Kos'va*) and Kan (*S. K-K*) plants, and a species of schizosaccharomycetes present in the yeast mass of a Leningrad hydrolysis plant (*S. b/u*) where saccharomycetes are used in the fermentation of woodwort.

Cultures of *S. pombe* 6-D and *S. acidodevoratus* were used as controls.

It should be noted that the *S. pombe* 6-D had a slightly lowered fermenting capacity, apparently as a result of storage under laboratory conditions.

When cultivated on commercial apple juice with 1.1 ml acidity and 7.72% sugar, *S. pombe* 6-D reduced acidity by 55%, while *S. acidodevoratus* and the test yeasts reduced it by 90%.

On synthetic medium with 0.8% and 1% malic acid the test yeasts reduced acidity in various degrees, but the greatest reduction was noted among the *S. acidodevoratus* yeasts (Fig. 1).

On media with 3% and 6% malic acid we observed a significant reduction of acidity by the *S. acidodevoratus* yeasts at the same time that the test yeasts brought about only a moderate reduction. The *S. acidodevoratus* yeasts continued to reduce acidity in the course of the second and third days (Fig. 2).

We also observed marked reduction of acidity by the *S. acidodevoratus* yeasts on woodwort with 6% malic acid (Fig. 3).

On synthetic media with 3%, 4%, 6%, and 8% glucose plus 0.8% malic acid, acidity fell in proportion to the utilization of sugar by all yeasts in the experiment.

Yeast activity in the experiment was tabulated according to the rate of sugar fermentation. *S. acidode-*

Table 1. Media Employed in the Tests on Utilization of Malic Acid by Schizosaccharomycetes

Media	pH	Acidity in ml of 0.1 N NaOH per ml of medium	Sugar in %
Apple juice	3.4	1.1	7.72
Synthetic medium with glucose + 0.8% malic acid	3.2	1.2	2.63
Synthetic medium with glucose + 1% malic acid	3.1	1.5	2.63
Synthetic medium with glucose + 3% malic acid	2.6	4.5	2.63
Synthetic medium with glucose + 6% malic acid	2.4	9.0	2.63
Wood hydrolyzate + 0.8% malic acid	3.0	1.5	3.38
Wood hydrolyzate + 6% malic acid	2.4	7.3	3.38

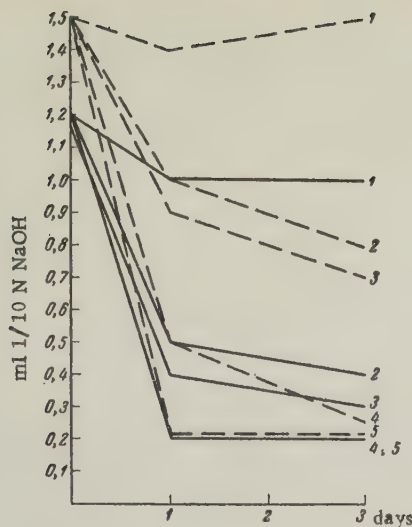


Fig. 1. Decline of acidity in synthetic medium with glucose plus 0.8% and 1% malic acid. — 0.8% malic acid; --- 1% malic acid. Designations in this and the following figures: 1) *S. pombe*; 2) *S. b/u*; 3) *S. Kos'va*; 4) *S. K-K*; 5) *S. acidodevoratus*.

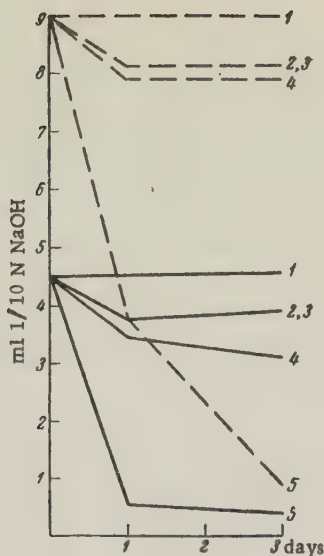


Fig. 2. Decline of acidity in synthetic medium with glucose plus 3% and 6% malic acid. — 3% malic acid; --- 6% malic acid.

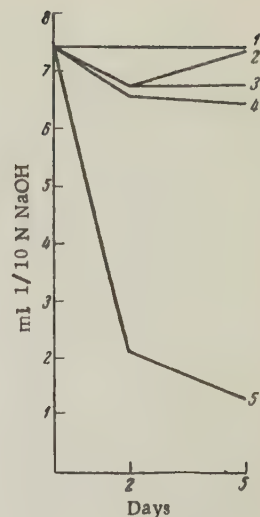


Fig. 3. Decline of acidity in woodwort with 6% malic acid content.

devoratus and the test yeasts fermented the sugar in 24 hours. Fermentation of sugar by *S. pombe* 6-D was retarded, apparently, because of its reduced activity after storage under laboratory conditions.

SUMMARY

1. Differentiation of the species of fission yeasts of the genus *Schizosaccharomyces* cannot be made with commercial apple juice that contains 1.1 ml of acidity.

2. A marked reduction of acidity by *S. acidodevoratus* yeasts was observed to take place on synthetic medium and woodwort with malic acid.

3. For species identification in the genus *Schizosaccharomyces* the synthetic medium we have indicated should be employed with 3% malic acid.

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*See English translation.

THE PHYSIOLOGY AND ECOLOGY OF THIOBACILLUS FERROOXIDANS IN RELATION TO ITS ROLE IN THE OXIDATION OF SULFIDE ORES (REVIEW)

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In 1947, Colmer and Hinkle (1947), who were studying acid drainage waters of coal mines, established the biological character of the oxidation of iron in these waters and isolated the organism responsible for this process. The isolated organism was found to be a motile, nonsporogenous rod, which differed from ordinary iron bacteria both in its morphology and in its ability to grow only in acid medium having a pH below 4. Taking into consideration the morphological similarity of the isolated organism to thiobacteria, as well as its ability to exist at the expense of the oxidation not only of iron but of hyposulfite as well, Temple and Colmer (1951) placed it in the genus *Thiobacillus* and suggested the name *Thiobacillus ferrooxidans*.

Further investigations of this organism showed that it has a large number of unique physiological properties, and that in addition to this, it plays a significant role in nature by participating in the oxidation of various sulfide minerals. In the present review, data are presented from the literature on the morphology, physiology, and ecology of *T. ferrooxidans* and its geochemical role.

THE MORPHOLOGY OF THIOBACILLUS FERROOXIDANS

T. ferrooxidans is a short rod with rounded ends, usually occurs singly, sometimes in pairs; reproduces by transverse fission; length 0.8-1.2 μ , width 0.4 μ ; motile, apparently by means of a single polar flagellum; Gram-negative. On agar with ferrous iron, it forms microscopic colonies encrusted with ferric hydroxide. According to the data of Colmer, Temple, and Hinkle (1949), the bacteria also produce fine colonies on agar medium containing hyposulfite; they are surrounded by a white substance (which is not sulfur) such as is secreted by *T. thioparvus* during growth on similar medium.

THE PHYSIOLOGY OF THIOBACILLUS FERROOXIDANS

One of the basic, theoretically important problems in the physiology of *T. ferrooxidans* was the question of the autotrophy of this organism. In order to resolve it, it was necessary to determine whether this organism can utilize carbon dioxide as the sole carbon source and can exist exclusively at the expense of en-

ergy obtained from the oxidation of ferrous iron. The solution of this question was especially important because the existence of autotrophic iron bacteria has been argued for many years, since Vinogradskii (1888, 1922) formulated his view of iron bacteria as a special group of microorganisms capable of activating the oxidation of ferrous iron and utilizing the energy released from this in their vital processes. *T. ferrooxidans* is an exceptionally convenient experimental material. In contrast to the majority of iron bacteria—which grow in neutral or slightly alkaline media, in which iron is easily oxidized by oxygen from the air, so that it is difficult to measure biological oxidation—this organism exists under conditions in which the chemical oxidation of iron is almost entirely absent. Thus, Leathen et al. (1956) found that the ferrous iron contained in acid mine waters in the amount of 200 mg/liter was oxidized by a culture of iron-oxidizing bacteria in three days, whereas more than two years would be required for the chemical oxidation of a sterile solution of iron by oxygen from the air at the same active acidity of the medium.

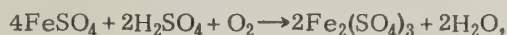
The first experiments on the physiology of *T. ferrooxidans* were carried out on cultures growing on simple synthetic medium which included magnesium and ammonium sulfate, as well as from 2 to 20 g/liter of iron sulfate. The pH of the medium was adjusted to 2-2.5 with sulfuric acid (Temple and Colmer, 1951). Thus, on this medium, there were three possibilities for obtaining energy: the utilization of organic impurities, the oxidation of ammonia, and the oxidation of iron. The possibility of existence at the expense of organic impurities was eliminated by using chemically pure salts and water which was twice distilled with permanganate. The vessels in which the experiments were carried out were cleaned with hot concentrated sulfuric acid. As for the utilization of ammonia for obtaining energy, first, this was prevented by very acid medium and second, according to calculations, the oxidation of all of the ammonia in the medium could not have given the increment of organic material observed in the experiment. Aside from this, the bacteria could grow, although more slowly, in medium in which nitrates were the only source of nitrogen.

T. ferrooxidans does not grow on organic media. Attempts to grow this organism on acid glucose peptone agar or in acid medium where the iron was

in the form of the butyrate, citrate, or lactate were not successful. According to the data of Leathen et al. (1956), many organic substances such as carbohydrates and peptone had an inhibitory effect on the oxidation of iron. Even impurities present in agar have an inhibitory effect, in connection with which it is nearly impossible to obtain colonies on agar medium. The effect of organic substances on the oxidation of pyrite by a pure culture of T. ferrooxidans was studied by Bryner and Jamerson (1958). They established that 4 g/liter of glucose or sucrose, as well as the addition of kerosene, had no effect on the process of pyrite oxidation. This process is significantly retarded in the presence of 1% acetone, and ceases entirely in a saturated solution of benzene. In our experiments (Lyalikova, 1959), 1% glucose also had no effect on the oxidation of iron, while sodium acetate and formate had strong toxic effects.

Carbon dioxide from the air is the only carbon source utilized by T. ferrooxidans. When air freed of carbon dioxide by means of alkali was passed through the medium, neither oxidation of iron nor growth of the cells was observed. Thus, T. ferrooxidans is an autotrophic organism which utilizes the energy from the oxidation of iron. The investigation of this new organism completely confirmed Vinogradskii's concept of the existence of autotrophic iron bacteria.

Temple and Colmer determined amounts of iron oxidized and carbon fixed. According to their data, 16.05 mg of carbon was fixed as the result of the oxidation of 120 g of iron sulfate. The calculation of the efficiency of energy utilization made by Temple and Colmer was based on the assumption that the oxidation of iron, occurring according to the following reaction:



gives 11.3 cal per 1 gram-atom, while the fixation of 1 gram-atom of carbon requires 115 kcal.

Temple and Colmer got 3.2 as the percent energy utilization. The ratio of weight of the biomass produced to iron oxidized, calculated on the basis of their data, was equal to 1:1155 and differed from the ratio 1:500 which, as proposed by Starkey (1945) on the basis of theoretical calculations, should be observed in cultures of autotrophic iron bacteria. In the experiments of Beck and Elsdon (1958), which were carried out in a Warburg apparatus, the molecular ratio of carbon dioxide fixed to iron oxidized was higher than in Temple and Colmer's experiments, and varied from 0.0045 to 0.01.

In our opinion, the low percent of energy utilization obtained by Temple and Colmer is explained by the fact that old 17-day cultures were used in their experiments in which the efficiency of energy utilization was much lower than in young cultures not older than two days. According to our data (Lyalikova, 1958), the percent of energy utilization in these cultures averaged 30. The most recent work of Silverman and Lundgren (1959) confirmed the data on the high efficiency of energy utilization by the T. ferrooxidans culture. In their experiments, which were carried

out in a Warburg apparatus, carbon dioxide was assimilated with an average efficiency of $20.5 \pm 4.3\%$.

A very interesting physiological property of T. ferrooxidans is its ability to utilize the lower compounds of sulfur, particularly hyposulfite, as an energy source. This ability distinguishes T. ferrooxidans from the majority of autotrophic bacteria, which utilize only one source of energy. Growth on hyposulfite was noted by Colmer, Temple, and Hinkle (1949). In their experiments, the bacteria oxidized hyposulfite to sulfuric acid, the secretion of which resulted in greatly reduced pH of the medium and the precipitation of sulfur due to the decomposition of hyposulfite. When transferred from the hyposulfite-containing medium to agar with ferrous iron, typical fine colonies with deposits of amber-colored iron appeared. The data of Colmer, Temple, and Hinkle concerning the oxidation of both iron and hyposulfite by a single species of bacteria did not find confirmation in the work of Leathen et al. (Leathen, Kinsel, and Braley, 1956). In Leathen's opinion, the oxidation of hyposulfite in Colmer and Temple's experiments could have been caused by purely chemical reactions. The organism which this author isolated from acid mine waters did not differ morphologically from T. ferrooxidans; it had the capacity to oxidize iron within the same pH range of 2-4.5, but did not grow on acid thiosulfate medium. Leathen and Braley (1954) placed this bacterium in the family Siderocapsaceae and called it Ferrobacillus ferrooxidans. Thus, the organisms described by Leathen and by Temple belong, according to Bergey's Manual (1957), not only to different genera, but even to different families; this is hardly expedient, since these organisms are probably identical. As pointed out by Temple, repeated transfers of T. ferrooxidans on iron-containing medium lead to the loss of the ability to oxidize hyposulfite, but cells growing on hyposulfite retain their ability to oxidize ferrous iron (Vishniac and Santer, 1957). But even if there is some doubt of the ability of iron-oxidizing bacteria to grow on hyposulfite, the experiments of a large number of authors carried out on minerals in which the only energy source could have been sulfide sulfur, proved beyond a doubt the ability of a single species of bacteria to utilize the lower compounds of both iron and sulfur (Bryner et al., 1954; Bryner and Anderson, 1957; Bryner and Jamerson, 1958). Bryner and Jamerson, as well as Silverman and Lundgren, report that they have cultures of iron-oxidizing bacteria which are capable of utilizing elemental sulfur. Although the first two authors feel that their culture is more closely related to T. ferrooxidans, while Silverman and Lundgren, working with Leathen's strain, relate their culture to Ferrobacillus ferrooxidans, these bacteria obviously belong to the same species and will henceforth be designated as Thiobacillus ferrooxidans.

The majority of work devoted to T. ferrooxidans pertains to the study of the effect of this organism on various sulfide minerals. In the experiments, sulfur-containing inclusions isolated from coal, such as pyrite and marcasite, as well as the sulfides of copper and of other metals, have been chiefly used.

Table 1. The Effect of *T. ferrooxidans* on Chalcopyrite

Time (days)	Amount dissolved, in mg			
	experimental		control	
	copper	iron	copper	iron
14	9.4	1.0	8.1	1.0
28	16.4	1.3	16.4	1.4
42	68.8	2.3	20.9	1.5
56	160.8	20.0	25.0	1.8
70	192.8	36.0	26.2	2.0
84	229.8	43.4	28.1	2.4

Table 2. The Biological Oxidation of a Mixture of Molybdenite with Pyrite

Time (days)	Amount dissolved, in mg			
	experimental		control	
	molybdenum	iron	molybdenum	iron
7	1.0	8.0	1.5	1.0
14	9.4	364.0	2.0	3.0
21	27.4	834.0	2.5	4.5
28	48.2	1304.0	2.9	6.0
35	72.1	1599.0	3.1	7.2
49	93.6	1793.0	3.3	9.0
63	100.1	1897.0	3.5	10.8
77	103.4	1913.0	3.6	13.0

Table 3. The Distribution of *T. ferrooxidans*

Deposits	Areas examined	Author, year
Coal	Bituminous coal mines in Pennsylvania and West Virginia, USA	Colmer, Temple, and Hinkle, 1949; Leathen, Braley, and McIntyre, 1953; Temple and Delchamps, 1953
	Coal mines in Scotland Lignite mines in Denmark Coal field near Moscow Lignite deposit in Kabarda Donetz coal field	Ashmeed, 1955 Fjordingstad, 1956 Lyalikova, 1959 " " Zarubina, Lyalikova, and Shmuk, 1959
Sulfide ores	Copper mines in Bingham Canyon, Utah, USA	Bryner et al., 1954
	Copper deposit in Cananea, Mexico Akhtala polymetallic deposit, Armenian SSR Degtyarsk pyrite deposit, Ural Mountains 3rd International pyrite deposit, San-Donato, Ural Mountains Krasnogvardeisk pyrite deposit, Ural Mountains Copper-nickel deposit, Nittis-Kumuzh'e, Kola Peninsula Copper-nickel deposit, Kaula, Kola Peninsula Rare-metal deposits of Central Kazakhstan	Bryner and Jamerson, 1958 Ivanov et al., 1958 Lyalikova, 1960 " " " " " " Kramarenko, 1960
Bodies of water	Ferruginous brook, Borok* in Yaroslavl Region Stream in neighborhood of coal field near Moscow Drill hole and lake at Gai health resort, Chelyabinsk Region	Lyalikova, 1959

*Transliteration of Russian—Publisher.

Leathen et al. (Leathen et al., 1953; Leathen, 1953) established that the bacteria isolated by them from the drainage waters of coal mines are capable of growing on marcasite and certain types of "sulfur balls"—inclusions in coal which are also iron disulfide. According to their data, pyrite was not affected by the bacteria. However, Temple and Delchamps (1953) demonstrated that a culture of *T. ferrooxidans* is capable of growing on pyrite as well, acting both on pyrite concretions from coal and on pure museum specimens of pyrite. Small particle size is a condition for the biological oxidation of pyrite. Bryner et al. (1954) conducted their experiments on the oxidation of sulfides in air lift percolators. These experiments confirmed the ability of *T. ferrooxidans* to oxidize pyrite. According to the data of Bryner et al., the amount of iron going into solution in the experimental percolator was 20 times greater than in the sterile control. When the percolators were placed in ice, the amount of oxidized iron decreased by 88% as compared with room temperature. The biological character of pyrite oxidation was also

confirmed by experiments in which heating and bactericidal substances were employed. The oxidation of chalcopyrite (CuFeS_2) due to the effect of bacteria is shown in Table 1.

The experimental percolator was inoculated only on the 28th day. Until this time, oxidation proceeded at an equal rate in both the experiment and in the control (Table 1). In the experiments of Bryner et al., aside from chalcopyrite, other copper sulfides were used as well. It was shown that the oxidation of covellite (CuS), chalcocite (Cu_2S), bornite (Cu_5FeS_4), and tetrahedrite ($\text{Cu}_8\text{SbO}_2\text{S}_7$) is accelerated by the action of *T. ferrooxidans*.

In subsequent works by Bryner and Anderson (1957) and Bryner and Jamerson (1958), pure cultures of *T. ferrooxidans* were used in experiments on the oxidation of sulfides. Through their action, about 0.6% of molybdenite (MoS_2) was oxidized in two weeks. Seven times more molybdenite went into solution in the experimental percolators than in the sterile control. Oxidation was especially intensified upon addition of pyrite

In this case, nearly 30 times more molybdenum went into solution due to the action of the bacteria than in the sterile control. Thus, laboratory experiments showed that T. ferrooxidans is capable of oxidizing ferrous iron and the sulfides of various metals. On the basis of these data, we can go on to a discussion of the geochemical role of this organism.

THE GEOCHEMICAL ROLE OF THIOBACILLUS FERROOXIDANS

Since the activity of T. ferrooxidans in nature can to some extent be judged by its distribution, data on the distribution of this organism are presented in Table 3. As seen from the table, the principal habitats of T. ferrooxidans are coal strata containing sulfide inclusions and deposits of sulfide ores. In the coal fields of the USA, the occurrence of T. ferrooxidans is connected with the area of distribution of acid drainage waters. This association is not casual. As shown by the laboratory investigations given above, the production of acid from the sulfur-containing inclusions of coal increases considerably through the action of bacteria. The laboratory experiments are confirmed by the observations of Temple and Delchamps on coal strata immediately after exposure. The reaction of the water in this segment was neutral, and T. ferrooxidans as well as T. thiooxidans were absent. However, after several days, the waters were already highly acid and these two species of bacteria were found in large numbers. The amount of acid drainage waters formed in mines with bituminous coal reaches tremendous volumes. Thus, according to Hodge's data (1937), in 1932 about 3 million tons of sulfuric acid (recalculated on the basis of the concentrated acid) was discharged with drainage waters into the Ohio River alone. The sulfuric acid produced causes corrosion of mining equipment and, getting into the river, the corrosion of dams and bridges. Ashmeed (1955), on the basis of experiments conducted, figures that 4/5 of all the acid is produced by biological means. When the data of Ashmeed and Hodge are compared, it becomes clear how great the work done by the bacteria is. The oxidation of ore in many sulfide deposits is also largely brought about by bacteria.

This pertains especially to deposits containing pyrite. The Degtyarsk pyrite deposit in Central Ural can serve as an example of a deposit in which the oxidative activity of bacteria plays a considerable role. The number of T. ferrooxidans in the mine waters of this deposit reached 10,000 cells/ml, while laboratory experiments showed that the bacteria speed up the oxidation of ore 8-10 times. Although bacteria evidently oxidize ore much more slowly under natural conditions, the high acidity of mine waters and the large amount of dissolved copper sulfate apparently is due largely to the oxidative activity of microorganisms. This activity can have practical significance as well. In specific cases, such as in the Akhtala deposit, for example, it is harmful, since the oxidation of ores in dumping grounds interferes with the separation of polymetallic ores in the process of flotation. In Cananea in Mexico, about 650 T of copper is additionally obtained per month

as the result of the leaching of tailings (Weed, 1956), chiefly due to bacterial oxidation. In addition to the direct effect of bacteria on sulfides, their ability to rapidly oxidize ferrous sulfate in acid solution can also be utilized; ferrous sulfate is formed when ferric sulfate solution is used for leaching sulfide ores. The utilization of bacteria makes it possible to provide continuous circulation of the leaching solution and simplifies the entire operation considerably. In the USA, a patent for this method was obtained by the Kennecott Copper Company (Zimmerley et al., 1958).

The production of metals from poor ores is of great significance at present, but will assume particular importance in the future when rich ore deposits will be largely exhausted. The bacterial enrichment of ores is one method of solving this problem.

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92 pages. Price: 1 ruble, 85 kopecks.

Reviewed by M. V. Fedorov.

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A Glossary of Terms for a Course in Agricultural Microbiology by A. I. Metelkin and O. A. Metelkin (a publication of the All-Union Agricultural Institute for Education by Correspondence) is a textbook intended for students of agronomy and zootechny at agricultural institutes. In view of the fact that this text contains a whole series of conspicuous errors in the definitions of relevant terms, it would be well to draw them to the attention of microbiologists who carry on the teaching programs at agricultural institutes. Below are cited several errors of a scientific nature which distort the meanings of the terms and therefore cannot be tolerated in a textbook.

1. Bacteria (p. 11), are defined by the authors as "1) The most extensive among all the groups (types) of microorganisms, which comprises microscopic creatures of plant origin, principally unicellular, lacking chlorophyll, reproducing by binary fission, and taxonomically divided into families of coccicoidal, bacilliform, spiral, and filamentous...." If we accept such a definition of this term, then the entire group of green and purple sulfur bacteria, the cells of which do contain chlorophyll, drop out altogether from microbiology. Such an attitude on the part of the authors towards this important group of bacteria evidently is explained by their ignorance of the literature dealing with these bacteria. This can be inferred from their interpretation of the term "photosynthesis" (p. 81). The authors of this manual define it as "the process of assimilation by plant organisms of carbon dioxide from air or water with its subsequent conversion into complex organic compounds in the presence of sunlight. Only organisms with chlorophyll or other pigments possess this faculty; but microorganisms, which do not possess them, make use of chemosynthesis instead of photosynthesis."

From this definition it also follows that among the bacteria there are no forms containing chlorophyll and capable of carrying on photosynthesis.

2. Lactobacteria (p. 37). In this section the authors give the following paragraph: "Lactolase is an enzyme which takes part in the subsequent course of lactic acid fermentation, and by its action glucose, galactose, and other monosaccharides are split up to form lactic acid." From this definition it appears that the non-existent enzyme lactolase splits up monosaccharides into lactic acid. All the achievements of science in the study of the chemical mechanism of fermentation have been liquidated here with one stroke of the pen.

3. Metabolism (p. 50). "Nutrition serves to build up the cell body from nutrients (preeminently nitrogen-

ous) ingested by the cell, accompanied by heat assimilation, i.e., it represents an endothermic reaction. Respiration, on the other hand, is intended to obtain for the cell the energy essential for its life processes from the material ingested during metabolism (preeminently carbohydrates) and is accompanied by the release of heat (exothermic reaction)." We have only to wonder how such "scientific" definitions of nutrition and respiration could have been published. If we are to proceed from these definitions, then it follows that we will accept that microorganisms consume and utilize heat and that it is this which accounts for their growth. Is this the definition of metabolism given at the present level of progress in the science of microbiology?

4. Silicate bacteria (p. 67), about which such plainly groundless information should not be included in a textbook of microbiology, are treated by the authors in the following way: "bacteria which are widely distributed in the soil and are distinguished for the versatility of their activity: They break down aluminum silicate, thereby releasing potassium; they secrete water-soluble phosphorite compounds; they assimilate atmospheric nitrogen; they stimulate the activity of soil microorganisms; they contribute to the reduction of plant morbidity." Here, it turns out, something truly unique has been encountered in the soil. There are enough of these bacteria alone to guarantee the nutrition of plants with all the radical elements and to dispel the agronomists' anxiety over crop yields. This entry on silicate bacteria, whose presence in nature so far has not been proven, can do nothing but harm. It deceives agronomists and places scholars in the position of fabulists.

5. Spirits (p. 70). "Wine or ethyl alcohol is produced by yeast fermentation of carbohydrates, resulting in the formation of ethanol (not over 16%), carbon dioxide, and water...." In this astonishing definition the authors make a new "revelation" in the field of biochemistry of microorganisms. Despite the findings of world science, they assert that in alcoholic fermentation not only ethanol and carbon dioxide are obtained as end products, but water as well. This "revelation" the authors pass on as common knowledge, not in the least troubled by the fact that it contradicts the findings of science.

6. Zoogleia (p. 28). — "an aggregate of bacteria which have the ability to form capsules (vide) and to secrete a mucus, like a natural glue or mucous colony." Surely the student will be utterly bewildered reading this definition of zoogleia, since he has not had occasion to see natural glue.

7. The cell envelope (p. 50) — "those of most bacteria are exceptionally thin, but also they are of great importance in regulating the passage of food material into the cell and the elimination of the products of its life processes." This definition also plainly does not correspond to reality, inasmuch as the external envelope does not regulate the penetration of substances into the cell and secretion from the cell into the surrounding medium. This function, as is well known, is fulfilled by the cytoplasmic membrane.

8. Cytoplasm (p. 83) — "the same as protoplasm." If these are one and the same thing then why do we

have two names? And, nevertheless, even from a microbiology textbook it is easy to see that they are not one and the same thing.

There is an enormous number of these inaccuracies in this textbook. Therefore, without entering into a detailed criticism, I should like to warn college microbiology instructors against accepting many of the definitions in the form set forth by the authors, inasmuch as they do not meet the fundamental requirements of scientific validity; nor do they reflect the present level of scientific knowledge in the field which they are attempting to clarify for students.

CONFERENCE ON THE PROBLEM OF PHYTONCIDES

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The third conference on the problem of the phytoncides, co-sponsored by the Institute of Microbiology, Academy of Sciences, UkrSSR, and the A. A. Zhdanov Leningrad University, was held in Kiev on June 22-25, 1959.

Approximately 200 participants contributed to the conference. Attending were specialists from various fields of study: biologists, microbiologists, botanists, agronomists, physicians, chemists, biochemists, etc., representing about 60 institutes for scientific research and training, as well as institutions of applied science from various cities in the Soviet Union: Baku, Biisk, Brest, Vinnitsa, Vil'nyus, Vladivostok, Voronezh, Dnepropetrovsk, Erevan, Kazan, Kamenets-Podol'sk, Kiev, Kishinev, Kemerov, Krasnoyarsk, Leningrad, Moscow, Nikolaev, Odessa, Ordzhonikidze, Orenburg, Perm, Pyatigorsk, Riga, Rostov-on-Don, Samarkand, Saratov, Stalinabad, Tartu, Tiflis, Khar'kov, Kherson, Yalta, and Jaroslaw.

The conference was opened by Academician V. G. Drobot'ko (Kiev), of the Academy of Sciences, UkrSSR, who expressed regret that the originator of the phytoncide inquiry, Tokin, could not be present at this conference.

In this address Drobot'ko described the present state of research on phytoncides and marked out the course for its further development. His principal attention was on isolation of antimicrobial substances in their pure form, disclosure of their chemical properties, and synthesis of derivatives with properties superior to the initial antimicrobial substances from plant sources.

At the conference, about 100 reports and scheduled papers were presented, representing the work of entire collectives as well as of individuals.

Several reports were devoted to the role of phytoncides in plant life: their importance in the formation of vegetative associations, their connection with plant vigor, with metabolism, etc. (Vlasyuk and Porutskii, Kiev; Chasovennaya and Khetagurova, Leningrad; Vysotskii, Biisk).

Several investigators presented results of studies on the intensity of formation of the volatile phytoncides depending on the growth cycle of plants, the effect of sunlight, the length of storage time (of fruits), etc. (Antadze and Chubinidze, Tiflis; Slavenas, Vil'nyus; Nikitina, Leningrad), and also of the phytoncide activity of seeds in dormancy and germination (Tul'-chinskaya, Odessa).

Sanadze (Tiflis) reported on an attempt to identify the chemical components of volatile phytoncides by

means of chromatography. A report was also made on the importance of aldehydes as components of the volatile products of several woody plants (Skvortsov, Leningrad).

Dzhaparidze (Tiflis) raised the question of the necessity for standardizing techniques for investigating volatile phytoncides. He suggested comparing phytoncide activity by its effect on infusoria, with a 0.01% formalin solution taken as the standard.

Considerable attention was given to reports on the significance of phytoncides in plant resistance to various diseases.

The leading address on plant immunity was given by Verderevskii (Kishinev). He expressed the opinion that if natural nonspecific immunity in animals has been confined primarily to phagocytosis throughout their evolutionary development, then, in plants, whose cells possess membranes, it is chiefly a matter of the extracellular excretion of the poisonous products of metabolism.

In their communications, a series of investigators from the Moldavian SSR (Verderevskaya, Voitovich, Dashkeeva, and Naidenova) illustrated this position with experimental material.

Reports were given on the inactivation of the tobacco mosaic virus with juices from tomatoes of unsusceptible varieties, on the bactericidal property of juice from the cotton plant immune to gummosis, and on the specific effect of the juice from grape leaves (of resistant varieties) on zoospores of the mildew pathogen. In the reports of Bel'tyukova (Kiev) and Galach'yan (Erevan) the importance of phytoncides was demonstrated as factors increasing the resistance of tomatoes and also of cabbage to their particular bacterioses.

Izrail'skii, Ryzhkova, and Rozanova (Moscow), while not denying the role of phytoncides in plant immunity, gave examples of the use of incorrect techniques (cultivation of seeds with sublimate) which had led some investigators into error, as a result of which mistaken conclusions were made on the existence in bean plants of specific immunity to various species of nodular bacteria.

Sorokina (Moscow) presented data demonstrating the role of phytoncides in selection of microorganisms of plant root systems.

A portion of the reports was devoted to the importance of phytoncides in the struggle of plants against a variety of harmful agents: leaf rusts, acacia scorch, etc. (Ocheretenko, Kamenets-Podol'sk; Fuga, Odessa).

An important place was occupied by reports devoted to the study and practical application of phytoncide preparations in controlling a variety of diseases of agricultural crops.

Thus, Bal'tyukova and Pastushenko (Kiev) obtained positive results from tests on the effect of an antibiotic preparation from wild hemp (kansatin 2) against potato bacterioses, and also in treatment of tomato seeds before planting. Bobyr' (Kiev) successfully applied imanin and arenarin against the tobacco mosaic virus.

Nikolaeva (Voronezh) investigated a series of preparations for control of corn mycoses (a preparation of garlic, onion, two species of clematis, and mustard, and also kansatin 2, arenarin, and stepin), and reached the conclusion that the phytoncides of many plants protect the seeding material and prevent diseases in the plants grown from it.

Cheremisinov and Vandysheva (Voronezh) also reported that they had successfully applied garlic phytoncides to control loose smut of millet, certain diseases of Siberian larch seedlings, and rotting of potatoes in storage.

Okazov (Ordzhonikidze) suggested ramson phytoncides as antimicrobial substances in the control of plant diseases and for preserving food products.

Grimm (Leningrad) reported on the successful application of extract from dry onion scale, radish, garlic, and horseradish, and also of mustard powder in the storage of carrots.

Many reports were devoted to the practical application in general and veterinary medicine of volatile phytoncides, vegetable antibiotics, and antibiotics of animal origin.

Papers were heard on the medicinal properties of the juice of archillea (Rusman, Riga), cranberry (Lekarenko, Krasnoyarsk), and aloe (Gerasimov, Nikolaev) in tuberculosis.

Several papers dealt with the application and further study of the antibiotic imanin, obtained by a group of workers at the Institute of Microbiology, Academy of Sciences, UkrSSR, from the common St.-John's-wort. The property of imanin to facilitate healing of severe burns and suppurating wounds was again confirmed, and its effectiveness was also shown in healing experimental radiation diseases of the skin. A case was presented of the successful application of imanin in anaerobic infection, as well as data demonstrating its anti-shock properties, and its activating effect on the ciliate epithelium of the upper respiratory tract (Suslov and Ivanov, Kiev; Pavolotskii, Vladivostok; Salant and Polunov, Odessa). The vegetable antibiotic imanin thus continues to attract the attention of investigators.

The attention of physicians is attracted primarily to garlic. In the form of a variety of preparations they have been using it with a wide range of diseases, from tuberculosis of the bone and acute cystitis to trichocephaliosis (Ets, Khodkevich, Grigor'ev, Gutman, and Lopatukhina, Jaroslaw; Ageeva, Rostov-on-Don).

Klimenko (Samarkand) carried out a large work on the application of dry garlic powder for healing sup-

purating wounds in patients and in an experiment with combined lesions (radiation sickness plus wounds of soft tissues); she recommended application of this preparation particularly in the first phase of the vulnerary process.

Tagiev (Baku) presented data on the favorable effect of garlic preparations which he used in the treatment of a variety of surgical diseases (over 500 cases), including tuberculous lymphadenitis.

Of interest, too, was the paper by Korotkov (Khar'kov), who in several thousand cases of the treatment and prophylaxis of influenza successfully used various garlic preparations for nose drops and for use with the "inhaphen" inhaler. It was all the more interesting that in the paper immediately following this Zelepukha (Kiev), who had examined experimentally the effect on the influenza virus of preparations from 22 plants, reported that in the literature there exists the opinion that garlic preparations do not affect the influenza virus. Garshin (Odessa) successfully treated angina and acute rhinitis, while Oks (Stalinabad) made use of volatile phytoncides of garlic in treating a series of diseases of the ear, nose, and throat.

New evidence of the medicinal properties of propolis was presented by workers from Kazan. They had successfully applied propolis in various medicinal forms for treatment of fresh and infected wounds, burns, and several skin diseases of agricultural animals, those afflicted with necrobacillosis in particular (Kazakov and Gaptrakhimanova). Milk supplemented with 5% propolis ("propolis milk") not only had medicinal properties, but even significantly stimulated growth of the animals (young swine).

A few papers were devoted to an experimental study of the efficacy and the mechanism of several vegetable antibiotics on the systems, organs, and functions of the animal organism; on the blood pressure of mammals, reflector activity of amphibians, nerve centers, thiol enzymes, and development of antibodies (Gurevich and Poplyko, Kagan and Nashchokina, Krasnoyarsk; Razumovich and Margolin, Brest; Epshtein and Shchigol', Kiev; Tretyak, Vinnitsa).

Several speakers touched on the hygienic importance of green plantations — their influence on ionization of air, on microflora of the air, and on the human organism (Lakhno, Petrov, Bobok, Dumanskii, Sverchkov, and Geikhman, Kiev; Komarova, Kemerovo) — as well as the hygienic significance of several algae in sanitary purification of cisterns (Subbotina and Titova, Dnepropetrovsk).

In comparison with the last conference, which took place three years ago, there was an increase in the number of reports devoted to the production and thorough study of purified and concentrated preparations.

Thus, Novotel'nov and Ezhov (Leningrad) reported on a new antibiotic, gordecin, which they had obtained from barley seed. Its characteristic property was its marked effect on the respiratory systems of plant and microbial cells.

Degtyareva (Yalta) had obtained several highly active crystalline preparations from myrtle and eucalypti and

she expressed the opinion that they were of polyphenol character.

Preparations from eucalypti were also studied by Pochinok (Kiev), who examined their detoxicating properties, and by a group of investigators from Rostov-on-Don — Yanovich, Gabrilovich, Kachar'yan, Kishkina, Mandrovskaya, and Semenova. They examined the significance of cineol in antituberculosis chemotherapy.

A group from Kiev—Derbentseva, Rabinovich, Bondarenko, Aizenman, Shvaiger, Zelepukha, and Mandrik—reported on several new plant antibiotics—salviol from *Salvia officinalis*, kansatin 2 from wild *Cannabis*, imanin A from *St.-John's-wort*—and they also studied the influence of light on antimicrobial activity of preparations from *St.-John's-wort*. They then reported on antimicrobial substances from several medicinal and food plants.

Drabkin and Dumova (Orenburg) reported on a preparation obtained from pelargonium leaves which had been sterilized in an autoclave. Rashba and Mostovova (Kiev) presented new data on the antibiotic arenarin, previously obtained from sandwort. Bedrosova (Leningrad) spoke on the antimicrobial properties of the anthocyanins of red cabbage, and Rotmistrov (Kiev) spoke on the antimicrobial and healing properties of several essential oils and their component substances. Tul'chinskaya (Odessa) examined the effect of a series of preparations on the *Brucellae*. Mamaichuk and Shinkarenko (Pyatigorsk) had studied the anti-

microbial properties of cyanophytin, a substance formed by algae of *Tambukan Lake*.

Kirsanov (Saransk) reported on an antimicrobial substance from the threads of the common synanthrous spider-flower, and Villako and Villako (Tartu) presented a detailed study of an antibiological substance they had obtained from broad flax. A paper was heard by Model' (Moscow) on the antimicrobial activity of tissue hydrolyzates.

Protopopov (Moscow) dealt briefly with the problem of synthesizing chemical derivatives of the known antimicrobial substances of plant origin, chaulmoogric and hydnocarpic acids.

The paper by Makhlayuk (Saratov) entitled, "The morphology of the volatile phytoncides" was exceptional. Microscope displays showed "crystalline" and "non-crystalline" formations which had settled on glass slides attached to test plants. A vigorous discussion followed on the nature of the formations.

Many valuable comments were made in speeches about the reports. Berezova (Moscow) remarked that there is an urgent need to give serious consideration to raw materials for preparations which have been or are intended to be put into production. Gerasimov (Moscow) reported on successful attempts at growing eucalypti in the environs of Moscow.

A resolution was taken at the conference calling for a series of measures to facilitate the further development of the problem. In particular, there was noted the necessity for the creation of an Institute of Phytoncides.

THE SLOVAK TYPE CULTURE COLLECTION OF BREWER'S YEASTS

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In the latter half of 1958 the Laboratory for the Slovak Type Culture Collection of Brewer's Yeasts was organized in Bratislava. The Laboratory was set up at the main base of operations to grow stock cultures for the Bratislava Brewery and for the Microbiological Laboratory of the Scientific-Research Institute of the Brewing Industry. The collecting station for stock cultures at the Bratislava Brewery is the newest, the most up-to-date, and the most complete in our republic. It has been operating since 1953; until then it had only served the needs of its own plant. The Microbiological Laboratory created at the Scientific-Research Institute has taken on the task of obtaining stock cultures for production needs and for work in scientific research.

Our most important problem at the start was to obtain a working culture (source) which would fulfill the specifications of the home plant and at the same time be suitable for other breweries. By means of selection, we obtained a physiological race with moderate fermenting ability, strength, and consistency which we designated Bratislava B-S. Our choice subsequently proved to be a successful one, since the properties of the new race satisfied requirements in many brews.

As early as the beginning of 1959, the Laboratory began to supply stock yeast cultures to several Slovakian breweries. In 1960 we supplied stock cultures to all breweries in the eastern half of our republic and have begun shipments to breweries in the western part of Czechoslovakia; at the present time we are receiving orders from abroad.

From the outset it was apparent that the Laboratory could not be entirely dependent upon a single race of mother yeasts and that we would have to select another strain with different characteristics, in order to satisfy a wider range of special requests and to enable breweries to produce two types of beer with a cutback in green beer. For this reason, research work is now directed toward obtaining the desired culture by selecting and adapting a variety of stock cultures from our collection.

The Laboratory maintains a large collection of stock cultures of brewer's yeasts, which consists of domestic cultures and is supplemented by cultures from abroad, the latter as a rule having been received through exchanges. There are 60 strains in the collection, grouped according to morphological and biochemical properties. A representative type has been chosen for each group.

Inasmuch as complex scientific research cannot be carried out on a large number of strains from a single group, the typical strains are adapted for these purposes. These representative types were picked several years ago, before the Laboratory was established.

In Czechoslovakia all the research work on the bottom yeast *Saccharomyces carlsbergensis* has been carried out with the following four races: No. 481 (formerly R 1), the representative type for the first group, which was obtained at the Smikhov Brewery in Prague; No. 4817 (formerly R 17), obtained from industrial yeasts of the BUDVAR brewery in Budejovice, which is the representative type for the second group; No. 4837 (formerly R 37) obtained at the brewery in Prague-Golesovice, which is the representative type for the third group; and No. 4811 (formerly R 11) obtained from yeasts at the Louna Brewery, which is the representative type for the fourth group.

The cultures are stored under conditions which ensure that the various races will retain their properties. Properties of the stored yeasts are examined annually on standard medium. Reproductive energy and fermenting capacity are tabulated according to the number of cells produced; average cell size is determined statistically; agglutination and precipitation indices are measured in the presence of acid and at neutral pH; the mash is analyzed for alcohol content, actual and apparent degrees of fermentation, and residual sugar. So far all strains have retained their initial properties with no noticeable deviations.

In addition to this collection of brewer's yeast cultures, the Laboratory has been setting up a larger collection of other cultures of yeasts and yeastlike microorganisms used for a wide variety of research purposes. In this collection we have both domestic and foreign cultures, a considerable number of the latter having been obtained from a yeast laboratory in Holland. There are approximately 550 cultures in this collection. We have conducted a large number of research projects with these cultures, and the results from these have already been published in part.

In press at the present time is An Atlas of Yeasts and Yeastlike Microorganisms in which are set forth in documented form the morphological characteristics of most of the cultures and their variations under the influence of medium and conditions. Most of the strains of yeasts and yeastlike microorganisms with which research works in Czechoslovakia have been carried out in the past have come from this collection and are kept there in reserve.

In the course of many years of research work on the pathogenic yeastlike microorganisms we have accumulated a large collection of these cultures. But inasmuch as the Laboratory for the Slovak Type Culture Collection of Brewer's Yeasts is primarily interested in industrial cultures, only strains of the non-pathogenic yeasts are kept in our collection. The pathogenic strains are kept in the research laboratory for the Dermatology Clinic of the Faculty of Medicine at Komenskii University, which operates in close contact with our laboratory. At the present time, typical yeast races are being picked which would be suitable for a thorough study including serology and immunology.

In addition to this, a large project is being conducted on the botanical investigation of the types of "Hemiascales" which have been occurring. For this purpose the Little Carpathian Region and Southern Slovakia have been systematically investigated.

From the large amount of material received after publication of the results the collection has gradually acquired new species, species which are rarely found, and the most representative types—yeastlike microorganisms of world-wide distribution.

The Laboratory also maintains a comparatively large collection of wine yeasts for purposes of scientific investigation. There is a larger collection of these cultures at the Scientific-Research Institute of Oenology and Viticulture at the Czechoslovak Academy of Agricultural Sciences in Bratislava. From the plant at Trenčín we obtain stock cultures of the bakery and distillery industries for research and teaching purposes. These cultures are also kept in our collection.

The Research Laboratory for the Slovak Type Culture Collection of Brewer's Yeasts plans to publish annual reports on its work, reports on selection of race types, information of the condition of the cultures kept in the collection, and various practical instructions. The first report, in the form of an information booklet, was published in 1959. In 1960 a report will be published with a complete catalogue of our yeast strains. These reports can be ordered from the Laboratory for the Slovak Type Culture Collection of Brewer's Yeasts, 15 Czabova Street, Bratislava, Czechoslovakia.

DEFENSE OF DISSERTATIONS

A. E. Kosmachev

Due to circumstances beyond our control, the translation of this article was not available at the time the journal was printed. It will be included in the English translation of "Mikrobiologiya," Vol. 29, No. 6, which will be published in May-June, 1961.

Publisher.



ALEKSANDR SEMENOVICH RAZUMOV

The Editorial Board of the Journal "Mikrobiologiya" and a Group of Associates at the Institute of Microbiology, Academy of Sciences, USSR, and at the Institute of Public and Community Hygiene, Academy of Medical Sciences, USSR

Translated from Mikrobiologiya, Vol. 29, No. 5, pp. 791-792, September-October, 1960

Microbiological science suffered a heavy loss with the death, early on July 4, 1960, of Professor Aleksandr Semenovich Razumov, Doctor of Biological Sciences, Head of the Sanitary Bacteriology Section at the Institute of Public and Communal Hygiene, Academy of Medical Sciences, USSR.

A. S. Razumov was born in 1894. In 1917 he completed his studies at the Faculty of Biological Sciences, Moscow State University. After graduating from the University, he worked as an assistant to Professor A. P. Artari at Shanyavskii University, and then with Professor L. I. Kursanov in the Department of Lower Plants at Moscow University and at the Technical College. Later on in his scientific work he was closely associated with Professor E. E. Uspenskii at the Scientific Institute for Fertilizers, NKTP, and at the Rublev Pumping Station of the Moscow Waterworks Trust.

During World War II, A. S. Razumov worked at the "VODGEO" Institute, and later, at the Institute of Public and Communal Hygiene, Academy of Medical Sciences, USSR.

A. S. Razumov's scientific research work was conducted in the fields of hydromicrobiology and soil microbiology.

In a joint work with N. P. Remezov, he initiated studies on the vertical distribution of the microorganisms in a soil profile according to ecological conditions, and later developed techniques by which he

was able to settle the highly controversial question of Azotobacter distribution in podzolic soils.

In the field of hydromicrobiology, his works are principally concerned with sanitation in water supply, although they have profound general theoretical significance.

His first work, devoted to the study of marine algae, was carried out under the supervision of Professor A. P. Artari.

Later on, A. S. Razumov took up the study of hydromicrobiology in connection with water supply. By this time, methods for counting phytoplankton on membrane filters were becoming the practice in hydrobiology investigations. A. S. Razumov used this technique in counting the bacterial populations of water. The modification he proposed for the direct microscopic count technique proved to be simpler and more accurate than earlier techniques, and has been widely used both here in the Soviet Union and abroad. He made a broad study of bacterial distribution in a variety of water sources and showed that even in clear waters their numbers are tens of thousands times greater than had previously been estimated with agar plate counts. Subsequently, A. S. Razumov and K. K. Barsov were the first to use membrane filters to count enteric bacilli, thus reducing analysis time from 2-3 days to 12 hours.

During World War II, A. S. Razumov remained in Moscow at the "VODGEO" Institute and, under difficult conditions, he carried on research in the study

and control of biological obstructions of thermopile instruments and conductor pipes at thermoelectric power plants.

In view of the influx of waste products from the new chemical industry, he developed new biological indicators of putrefaction in reservoirs.

All these investigations were written up in the form of a monograph, "Microbial plankton," and in a series of other works.

The final years of his work at the Institute of Public and Communal Hygiene were devoted to studying problems of the ecology and interrelations of the bacterial populations and phytoplankton of reservoirs. He showed that during their lifetime the phytoplankton secrete substances which have an antagonistic effect on saprophytic microflora, whereas after the phytoplankton have died out, these substances stimulate its growth.

These works are of fundamental value for comprehending the life of aquatic organisms and for a knowledge of the self-purification processes of water in reservoirs. During the period of his research activi-

ties A. S. Razumov published approximately 70 scientific works.

From the earliest beginnings of the journal "Mikrobiologiya," he was one of its leading figures. At first, as Assistant to the Editor-in-Chief, Professor E. E. Uspenskii, he took an active part in editing manuscripts, and in recent years he was a Member of the Editorial Board. Throughout his research career, A. S. Razumov made a study of the foreign and Russian literature and he published critical articles in "Mikrobiologiya" and subsequently in the review "Biologiya."

A keen experimentalist, commanding a wide knowledge of the literature, he readily helped all who came to him with problems in hydromicrobiology. A. S. Razumov made an important contribution to the training of a highly qualified corps of microbiologists.

The services of A. S. Razumov to Soviet science were highly valued by the Party and by the Government, who awarded him the Order of Lenin and various medals.

His personal modesty and outstanding scholarship will long be remembered by his students and by all who came into contact with him.

HENRIETTA MOISEEVNA KUBLANOVSKAYA

A. A. Imshenetskii, E. I. Kvasnikov, T. M. Kondrat'eva, A. I. Korenyako,
N. A. Krasil'nikov, M. A. Litvinov, M. N. Meisel', Ya. I. Rautenshtein

Translated from Mikrobiologiya, Vol. 29, No. 5, p. 793, September-October, 1960

On April 3, 1960, the death of Henrietta Moiseevna Kublanovskaya, Assistant Professor of Agricultural Microbiology and Plant Physiology at the Tashkent Agricultural Institute, occurred. A painful illness has cut short the work of a talented and enthusiastic microbiologist.

Henrietta Moiseevna Kublanovskaya was born in 1905. She began active research work immediately after graduation from the Crimean University, where she specialized in microbiology; during the first seven years she worked in the Crimea as a laboratory assistant, and then as a microbiological research assistant, mainly at the Agricultural Conservation Stations and in the Agricultural Conservation Section of VILAR. After moving to Leningrad in 1932, she worked in the Laboratory of Microbiology of the Academy of Sciences, USSR until 1934, when this laboratory was transferred to Moscow. From 1934 until the beginning of World War II, H. M. Kublanovskaya worked first as Junior and then as Senior Research Assistant at the Microbiological Laboratory of the P. F. Lesgaft State Scientific-Research Institute. In 1939 she defended her candidate's dissertation on the subject: "The influence of osmotic pressure of the medium on the physiological properties of yeasts."

During World War II, Henrietta Moiseevna took up work in the field of medical microbiology, working in Leningrad in 1941-1942, at the time of the blockade, and then, after evacuation, in the Medical Sanitary Service of the Tashkent Vertical Textile Combine.

In 1947, Henrietta Moiseevna returned to scientific research as Senior Research Assistant at the Agricultural Conservation Station of SNIKHI (now the Agricultural Conservation Institute of the Uzbek Academy of Agricultural Sciences), where she studied the important and little-investigated biological methods for combatting verticillium wilt of cotton. At first, Henrietta Moiseevna examined the effect of mycolytic bacteria and their complexes on the cause of wilt, the fungus *Verticillium dahliae*.

H. M. Kublanovskaya gathered valuable data on the interrelations between the complexes and the individ-

ual species of mycolytic bacteria in the soils of Central Asia. Her particular attention was focused on the actinomycetes, and she developed several methods for isolating actinomycetes from the soil. Of special interest are her investigations in the search for growth stimulants of actinomycetes. The works of Henrietta Moiseevna devoted to the problem of combatting verticillium disease of cotton by means of antagonistic actinomycetes have received wide recognition. The methods she proposed were approved in industrial trials in a series of regions in Uzbekistan and Tadzhikistan and have now been successfully introduced into many provinces of China; as the author of this technique, H. M. Kublanovskaya participated in the All-Union Agricultural Exhibition of 1954.

During the past 11 years, H. M. Kublanovskaya was engaged in teaching work at the State University of Central Asia and at the Tashkent Agricultural Institute. Henrietta Moiseevna commanded a large scope of information, which she was able to pass on to her students with exceptional teaching skill.

She was the author of A Guide to Practical Studies in Agricultural Microbiology, and A Working Notebook, which were published in Tashkent and which offer considerable interest to instructors of agricultural microbiology at universities and technical colleges.

Henrietta Moiseevna assembled a large collection of actinomycetes, numbering more than 2000 strains, obtained from the soils of various zones in Central Asia.

All who came into contact with H. M. Kublanovskaya were aware of her clarity of purpose and dedication to scientific work.

Henrietta Moiseevna enjoyed great respect and affection as a scholarly, considerate, and sympathetic human being and teacher.

The memory of her as an outstanding scholar, a delightful person, and a remarkable teacher will long live in the hearts of her numerous comrades, friends, and students.

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED
IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
IKhN	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKht	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKHo	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Apteknoe Delo	Apteknoe Delo	Pharmaceutical Transactions
Ark. Anat. Gistol. i Émbriol.	Ark. Anatomií Gistologii i Émbriologii	Archives of Anatomy, Histology, and Embryology
Ark. Biol. Nauk SSSR	Ark. Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Ark. Patol.	Ark. Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologicheskii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatrica	Pediatrica	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN transliteration system.

(continued)

(continued)

Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheski Arkhiv	Therapeutic Archives
Trudy Gel'mint. Lab.	Trudy Gel'mintologicheskoi Laboratorii	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologii	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologii	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologii, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnyi Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologii	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologii	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirurgii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikiat.	Zhurnal Nevropatologii i Psikiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologii	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatel'nosti imeni L. P. Pavlova	L. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

AIBS Russian Monograph Translations

The AIBS is in the process of expanding its Russian Translations Program extensively. Funds to subsidize translation and publication of important Russian literature in biology have been obtained from the National Science Foundation, as part of a larger program to encourage the exchange of scientific information between the two countries. The following monographs have been published:

Origins of Angiospermous Plants. *By A. L. Takhtajan.*

Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.

68 pp. \$3.00, individuals and industrial libraries (U.S.A. and Canada);
\$2.00, AIBS members and all other libraries; \$.50, additional, foreign.

Essays on the Evolutionary Morphology of Plants. *By A. L. Takhtajan.*

Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.

144 pp. \$5.00, individuals and industrial libraries (U.S.A. and Canada);
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Problems in the Classification of Antagonists of Actinomycetes. *By G. F. Gause.*

Edited by David Gottlieb. Translated by Fritz Danga.

\$5.00, individuals and industrial libraries (U.S.A. and Canada);
\$4.00, AIBS members and all other libraries; \$.50, additional, foreign.

Arachnida. Vol. IV, No. 2. Fauna of the U.S.S.R. *By B. I. Pomerantzev.*

Edited by George Anastos. Translated by Alena Elbl.

\$10.00, individuals and industrial libraries (U.S.A. and Canada);
\$9.00, AIBS members and all other libraries; \$1.00, additional, foreign.

Arachnoidea. Vol. VI, No. 1. Fauna of the U.S.S.R. *By A. A. Zachvatkin.*

Translated and edited by A. Ratcliffe and A. M. Hughes.

\$10.00, individuals and industrial libraries (U.S.A. and Canada);
\$9.00, AIBS members and all other libraries; \$1.00, additional, foreign.

Marine Biology. Vol. XX, Trudy Institute of Oceanology. *Edited by B. N. Nikitin.*

\$10.00, individuals and industrial libraries (U.S.A. and Canada);
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DOKLADY

BIOLOGICAL SCIENCES SECTIONS

Dr. Charles C. Davis, Advisory Editor

6 issues per year. First issues published: 1957, Vols. 112-117. Current issues published: 1960, Vols. 130-135.

This is a translation of all papers in the Biological Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections in many areas of biology, among which are the following: biophysics, cytology, embryology, experimental morphology, genetics, histology, hydrobiology, microbiology, morphology, physiology, and zoology.

YEARLY SUBSCRIPTIONS: \$20.00, individuals and industrial libraries (U.S. & Can.); \$15.00, AIBS members and all other libraries; \$2.50 additional to each price, foreign. SINGLE COPIES: \$4.00.

DOKLADY

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This journal is a translation of all papers in the Botanical Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections on botany, plant anatomy, plant embryology, plant morphology, and plant physiology.

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This is a translation of all papers in the Biochemistry Section of the Proceedings of the Academy of Sciences of the USSR. These papers deal with all phases of the chemistry of plant and animal life, at both the biological and physiological levels. This translation contains a list of abbreviations most frequently encountered in Russian Bio-sciences literature, as well as a reference list of Russian journals that are frequently cited.

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Dr. Arthur W. Galston, Advisory Editor

Dr. Bruce A. Bonner, Asst. Advisory Editor

6 issues per year. First issues published: 1957, Vol. 4, Nos. 1-6. Current issues published: 1960, Vol. 7, Nos. 1-6.

This journal publishes original studies of an experimental nature on various problems of the physiology of plants, reviews, critical articles, and discussions of major problems in this field. It has special sections dealing with methods of experimental research and the application of finished scientific research to current agricultural practice.

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MICROBIOLOGY

(MIKROBIOLOGIYA)

Dr. J. R. Porter, Advisory Editor

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This journal deals with problems of general microbiology (systematics, morphology, physiology, and variability of microorganisms), agricultural microbiology (microbiology of soils and fodder crops, bacterial fertilizers), technical microbiology, the microbiology of foods, and the microbiology of water reservoirs. This journal also contains sections on the history of science, book reviews, and current events in the field of microbiology, as well as the general articles in this field.

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